1 **Chimeric binding peptide library screening method**

2

3 The present invention relates generally to methods for

4 screening nucleotide libraries for sequences that

5 encode peptides of interest.

6

7 Isolating an unknown gene which encodes a desired

8 peptide from a recombinant DNA library can be a

9 difficult task. The use of hybridisation probes may

10 facilitate the process, but their use is generally

11 dependent on knowing at least a portion of the sequence

12 of the gene which encodes the protein. When the

13 sequence is not known, DNA libraries can be expressed

14 in an expression vector, and antibodies have been used

15 to screen for plaques or colonies displaying the

16 desired protein antigen. This procedure has been useful

17 in screening small libraries, but rarely occurring

18 sequences which are represented in less than about 1 in

19 10^5 clones (as is the case with rarely occurring cDNA

20 molecules or synthetic peptides) can be easily missed,

21 making screening libraries larger than 10^6 clones at

22 best laborious and difficult. Methods designed to

23 address the isolation of rarely occurring sequences by

24 screening libraries of 10^6 clones have been developed

25 and include phage display methods and LacI fusion phage

26 display, discussed in more detail below.

27

28 Phage display methods. Members of DNA libraries which

29 are fused to the N-terminal end of filamentous

30 bacteriophage pIII and pVIII coat proteins have been

31 expressed from an expression vector resulting in the

1 display of foreign peptides on the surface of the phage

2 particle with the DNA encoding the fusion protein

3 packaged in the phage particle (Smith G. P., 1985,

4 Science 228: 1315-1317). The expression vector can be

5 the bacteriophage genome itself, or a phagemid vector,

6 into which a bacteriophage coat protein has been

7 cloned. In the latter case, the host bacterium,

8 containing the phagemid vector, must be co-infected

9 with autonomously replicating bacteriophage, termed

10 helper phage, to provide the full complement of

11 proteins necessary to produce mature phage particles.

12 The helper phage normally has a genetic defect in the

13 origin of replication which results in the preferential

14 packaging of the phagemid genome. Expression of the

15 fusion protein following helper phage infection, allows

16 incorporation of both fusion protein and wild type coat

17 protein into the phage particle during assembly.

18 Libraries of fusion proteins incorporated into phage,

19 can then be selected for binding members against

20 targets of interest (ligands). Bound phage can then be

21 allowed to reinfect *Escherichia coli* (*E. coli*) bacteria

22 and then amplified and the selection repeated,

23 resulting in the enrichment of binding members

24 (Parmley, S. F., & Smith, G. P. 1988., Gene 73: 305-

25 318; Barrett R. W. *et al.*, 1992, Analytical

26 Biochemistry 204: 357-364 Williamson *et al.*, Proc.

27 Natl. Acad. Sci. USA, 90: 4141-4145; Marks *et al.*,

28 1991, J. Mol. Biol. 222: 581-597).

29

30 Several publications describe this method. For example,

31 US Patent No 5,403,484 describes production of a

1   chimeric protein formed from the viral coat protein and

2   the peptide of interest. In this method at least a

3   functional portion of a viral coat protein is required

4   to cause display of the chimeric protein or a processed

5   form thereof on the outer surface of the virus. In

6   addition, US Patent No 5,571,698 describes a method for

7   obtaining a nucleic acid encoding a binding protein, a

8   key component of which comprises preparing a population

9   of amplifiable genetic packages which have a

10   genetically determined outer surface protein, to cause

11   the display of the potential binding domain on the

12   outer surface of the genetic package. The genetic

13   packages are selected from the group consisting of

14   cells, spores and viruses. For example when the

15   genetic package is a bacterial cell, the outer surface

16   transport signal is derived from a bacterial outer

17   surface protein, and when the genetic package is a

18   filamentous bacteriophage, the outer surface transport

19   signal is provided by the gene pIII (minor coat

20   protein) or pVIII (major coat protein) of the

21   filamentous phage.

22

23   WO-A-92/01047 and WO-A-92/20791 describe methods for

24   producing multimeric specific binding pairs, by

25   expressing a first polypeptide chain fused to a viral

26   coat protein, such as the gene pIII protein, of a

27   secreted replicable genetic display package (RGDP)

28   which displays a polypeptide at the surface of the

29   package, and expressing a second polypeptide chain of

30   the multimer, and allowing the two chains to come

31   together as part of the RGDP.

1

2 <u>LacI fusion plasmid display</u>. This method is based on

3 the DNA binding ability of the lac repressor. Libraries

4 of random peptides are fused to the lacI repressor

5 protein, normally to the C-terminal end, through

6 expression from a plasmid vector carrying the fusion

7 gene. Linkage of the LacI-peptide fusion to its

8 encoding DNA occurs via the lacO sequences on the

9 plasmid, forming a stable peptide-LacI-peptide complex.

10 These complexes are released from their host bacteria

11 by cell lysis, and peptides of interest isolated by

12 affinity purification on an immobilised target. The

13 plasmids thus isolated can then be reintroduced into *E.*

14 *coli* by electroporation to amplify the selected

15 population for additional rounds of screening (Cull, M.

16 G. et al. 1992. Proc. Natl. Acad. Sci. U.S.A. 89:1865-

17 1869).

18

19 US Patent No 5498530 describes a method for

20 constructing a library of random peptides fused to a

21 DNA binding protein in appropriate host cells and

22 culturing the host cells under conditions suitable for

23 expression of the fusion proteins intra-cellularly, in

24 the cytoplasm of the host cells. This method also

25 teaches that the random peptide is located at the

26 carboxy terminus of the fusion protein and that the

27 fusion protein-DNA complex is released from the host

28 cell by cell lysis. No method is described for the

29 protection of the DNA from degradation once released

30 from the lysed cell. Several DNA binding proteins are

31 claimed but no examples are shown except lacI.

1

2   There remains a need for methods of constructing

3   peptide libraries in addition to the methods described

4   above. For instance, the above methods do not permit

5   production of secreted peptides with a free carboxy

6   terminus. The present invention describes an

7   alternative method for isolating peptides of interest

8   from libraries and has significant advantages over the

9   prior art methods.

10

11 In general terms, the present invention provides a

12 method for screening a nucleotide library (usually a

13 DNA library) for a nucleotide sequence which encodes a

14 target peptide of interest. The method involves

15 physically linking each peptide to a polynucleotide

16 including the specific nucleotide sequence encoding

17 that peptide. Linkage of a peptide to its encoding

18 nucleotide sequence is achieved via linkage of the

19 peptide to a nucleotide binding domain. A bifunctional

20 chimeric protein with a nucleotide binding domain and a

21 library member or target peptide (preferably with a

22 function of interest) is thus obtained. The peptide of

23 interest is bound to the polynucleotide encoding that

24 peptide via the nucleotide binding domain of the

25 chimeric protein.

26

27 The polynucleotide-chimeric protein complex is then

28 incorporated within a peptide display carrier package

29 (PDCP), protecting the polynucleotide from subsequent

30 degradation, while displaying the target peptide

1 portion on the outer surface of the peptide display

2 carrier package (PDCP).

3

4 Thus, in one aspect, the present invention provides a

5 peptide display carrier package (PDCP), said package

6 comprising a polynucleotide-chimeric protein complex

7 wherein the chimeric protein has a nucleotide binding

8 portion and a target peptide portion, wherein said

9 polynucleotide comprises a nucleotide sequence motif

10 which is specifically bound by said nucleotide binding

11 portion, and wherein at least the chimeric protein

12 encoding portion of the polynucleotide not bound by the

13 nucleotide binding portion of the chimeric protein is

14 protected.

15

16 In one embodiment the polynucleotide is protected by a

17 protein which binds non-specifically to naked

18 polynucleotide. Examples include viral coat proteins,

19 many of which are well-known in the art. Where the

20 chosen viral coat protein requires an initiation

21 sequence to commence general binding to the

22 polynucleotide, this will be provided on the

23 polynucleotide at appropriate location(s). A preferred

24 coat protein is coat protein from a bacteriophage,

25 especially M13.

26

27 Generally, the nucleic binding portion of the chimeric

28 protein is selected for its specificity for the

29 nucleotide sequence motif present in the recombinant

30 polynucleotide encoding the chimeric protein.

31

1 Optionally, the nucleotide sequence motif may be an
2 integral part of the protein encoding region of the
3 polynucleotide. Alternatively, and more usually, the
4 motif may be present in a non-coding region of the
5 polynucleotide. For the purposes of this invention,
6 all that is required is for the motif to be located on
7 the polynucleotide such that the nucleotide binding
8 portion of the chimeric protein is able to recognise
9 and bind to it. Desirably the polynucleotide-chimeric
10 protein complex has a dissociation constant of at least
11 one hour.

12

13 Optionally, the recombinant polynucleotide may comprise
14 two or more nucleotide sequence motifs, each of which
15 will be bound by a chimeric protein molecule.
16 Preferably, the motifs are positioned along the length
17 of the polynucleotide to avoid steric hindrance between
18 the bound chimeric proteins.

19

20 Preferably, the nucleotide sequence motif is not
21 affected by the presence of additional nucleotide
22 sequence (e.g. encoding sequence) at its 5' and/or 3'
23 ends. Thus the chimeric fusion protein may include a
24 target peptide portion at its N terminal end, at its C
25 terminal end or may include two target peptide portions
26 (which may be the same or different) at each end of the
27 nucleotide binding portion, ie at both the N and C
28 terminal ends of the chimeric protein. For example one
29 target peptide may be an antibody of known specificity
30 and the other peptide may be a peptide of potential
31 interest.

1

2 Desirably the target peptide portion of the chimeric

3 protein is displayed externally on the peptide display

4 carrier package, and is thus available for detection,

5 reaction and/or binding.

6

7 In more detail the PDCP may be composed two distinct

8 elements:

9   a. A polynucleotide-chimeric protein complex. This

10   links the displayed target peptide portion to the

11   polynucleotide encoding that peptide portion

12   through a specific polynucleotide binding portion.

13   The nucleotide sequence encoding the chimeric

14   protein, and the specific nucleotide sequence

15   motif recognised by the nucleotide binding portion

16   of the chimeric protein must be present on a

17   segment of polynucleotide which can be

18   incorporated into the PDCP; and

19   b. A protective coat. This may be supplied by a

20   replicable carrier or helper package capable of

21   independent existence. Alternatively, a coat

22   protein could be encoded by the recombinant

23   polynucleotide of the invention. The protective

24   coat for the polynucleotide-chimeric protein

25   complex may be composed of a biological material

26   such as protein or lipid, but the protective coat

27   is not required for linking the target peptide to

28   the polynucleotide encoding that peptide. The

29   protective coat must allow the display of the

30   target peptide portion of the chimeric protein on

31   its outer surface. The carrier or helper package

1   may also provide the mechanism for releasing the

2   intact PDCP from host cells when so required. By

3   way of example, when a bacteriophage is the

4   replicable carrier package, a protein coat of the

5   bacteriophage surrounds the polynucleotide-

6   chimeric protein complex to form the PDCP, which

7   is then extruded from the host bacterial cell.

8

9   The invention described herein demonstrates that

10   peptides fused to a nucleotide binding domain can be

11   displayed externally, even through a bacteriophage

12   carrier package protein coat, while still bound to the

13   polynucleotide encoding the displayed peptide.

14

15   The present invention also provides a recombinant

16   polynucleotide comprising a nucleotide sequence

17   encoding a chimeric protein having a nucleotide binding

18   portion operably linked to a target peptide portion,

19   wherein said polynucleotide includes a specific

20   nucleotide sequence motif which is bound by the

21   nucleotide binding portion of said chimeric protein and

22   further encoding a non-sequence-specific nucleotide

23   binding protein.

24

25   Desirably, the recombinant polynucleotide is a

26   recombinant expression system, able to express the

27   chimeric protein when placed in a suitable environment,

28   for example a compatible host cell. After its

29   expression, the chimeric protein binds to the specific

30   nucleotide sequence (motif) present in the

1　polynucleotide comprising the nucleotide sequence

2　encoding the chimeric protein.

3

4　Optionally there may be a linker sequence located

5　between the nucleotide sequence encoding the nucleotide

6　binding portion and the polynucleotide inserted into

7　the restriction enzyme site of the construct.

8

9　Desirably the nucleotide binding portion is a DNA

10　binding domain of an [oestrogen] estrogen or

11　progesterone receptor, or a functional equivalent

12　thereof.　Examples of sequences encoding such

13　nucleotide binding portions are set out in SEQ ID Nos

14　11 and 13.

15

16　The term "expression system" is used herein to refer to

17　a genetic sequence which includes a protein-encoding

18　region and is operably linked to all of the genetic

19　signals necessary to achieve expression of that region.

20　Optionally, the expression system may also include

21　regulatory elements, such as a promoter or enhancer to

22　increase transcription and/or translation of the

23　protein encoding region or to provide control over

24　expression.　The regulatory elements may be located

25　upstream or downstream of the protein encoding region

26　or within the protein encoding region itself.　Where

27　two or more distinct protein encoding regions are

28　present these may use common regulatory element(s) or

29　have separate regulatory element(s).

30

1   Generally, the recombinant polynucleotide described

2   above will be DNA. Where the expression system is

3   based upon an M13 vector, usually the polynucleotide

4   binding portion of the expressed chimeric portion will

5   be single-stranded DNA. However, other vector systems

6   may be used and the nucleotide binding portion may be

7   selected to bind preferentially to double-stranded DNA

8   or to double or single-stranded RNA, as convenient.

9

10   Additionally the present invention provides a vector

11   containing such a recombinant expression system and

12   host cells transformed with such a recombinant

13   expression system (optionally in the form of a vector).

14

15   Whilst the recombinant polynucleotide described above

16   forms an important part of the present invention, we

17   are also concerned with the ability to screen large

18   (e.g. of at least 10^5 members, for example 10^6 or even

19   10^7 members) libraries of genetic material. One of the

20   prime considerations therefore is the provision of a

21   recombinant genetic construct into which each member of

22   said library can individually be incorporated to form

23   the recombinant polynucleotide described above and to

24   express the chimeric protein thereby encoded (the

25   target peptide of which is encoded by the nucleotide

26   library member incorporated into the construct).

27

28   Thus viewed in a further aspect the present invention

29   provides a genetic construct or set of genetic

30   constructs comprising a polynucleotide having a

31   sequence which includes:

1

2　　i)　　a sequence encoding a nucleotide binding portion

3　　　　　able to recognise and bind to a specific sequence

4　　　　　motif;

5　　ii)　　the sequence motif recognised and bound by the

6　　　　　nucleotide binding portion encoded by (i);

7　　iii)　a restriction enzyme site which permits insertion

8　　　　　of a polynucleotide, said site being designed to

9　　　　　operably link said polynucleotide to the sequence

10　　　　　encoding the nucleotide binding portion so that

11　　　　　expression of the operably linked polynucleotide

12　　　　　sequences yields a chimeric protein; and

13　　iv)　a sequence encoding a nucleotide binding protein

14　　　　　which binds non-specifically to naked

15　　　　　polynucleotide.

16

17　Optionally there may be a linker sequence located

18　between the nucleotide sequence encoding the nucleotide

19　binding portion and the sequence of the polynucleotide

20　from the library inserted into the restriction enzyme

21　site of the construct.

22

23　Desirably the nucleotide binding portion is a DNA

24　binding domain of an [oestrogen] estrogen or

25　progesterone receptor, or a functional equivalent

26　thereof. Examples of sequences encoding such

27　nucleotide binding portions are set out in SEQ ID Nos

28　11 and 13.

29

30　Suitable genetic constructs according to the invention

31　include pDM12, pDM14 and pDM16, deposited at NCIMB on

1   28 August 1998 under Nos NCIMB 40970, NCIMB 40971 and

2   NCIMB 40972 respectively.

3

4   It is envisaged that a conventionally produced genetic

5   library may be exposed to the genetic construct(s)

6   described above. Thus, each individual member of the

7   genetic library will be separately incorporated into

8   the genetic construct and the library will be present

9   in the form of a library of recombinant polynucleotides

10   (as described above), usually in the form of vectors,

11   each recombinant polynucleotide including as library

12   member.

13

14   Thus, in a further aspect, the present invention

15   provides a library of recombinant polynucleotides (as

16   defined above) wherein each polynucleotide includes a

17   polynucleotide obtained from a genetic library and

18   which encodes the target peptide portion of the

19   chimeric protein expressed by the recombinant

20   polynucleotide.

21

22   Optionally, the chimeric protein may further include a

23   linker sequence located between the nucleotide binding

24   portion and the target peptide portion. The linker

25   sequence will reduce steric interference between the

26   two portions of the protein. Desirably the linker

27   sequence exhibits a degree of flexibility.

28

29   Also disclosed are methods for constructing and

30   screening libraries of PDCP particles, displaying many

31   different peptides, allowing the isolation and

1 identification of particular peptides by means of
2 affinity techniques relying on the binding activity of
3 the peptide of interest. The resulting polynucleotide
4 sequences can therefore be more readily identified, re-
5 cloned and expressed.
6
7 A method of constructing a genetic library, said method
8 comprising:
9
10 a)   constructing multiple copies of a recombinant
11   vector comprising a polynucleotide sequence which
12   encodes a nucleotide binding portion able to
13   recognise and bind to a specific sequence motif
14   (and optionally also including the specific
15   sequence motif);
16
17 b)   operably linking each said vector to a
18   polynucleotide encoding a target polypeptide, such
19   that expression of said operably linked vector
20   results in expression of a chimeric protein
21   comprising said target peptide and said nucleotide
22   binding portions; wherein said multiple copies of
23   said operably linked vectors collectively express
24   a library of target peptide portions;
25
26 c)   transforming host cells with the vectors of step
27   b);
28
29 d)   culturing the host cells of step c) under
30   conditions suitable for expression of said
31   chimeric protein;

1

2   e)   providing a recombinant polynucleotide comprising

3   the nucleotide sequence motif specifically

4   recognised by the nucleotide binding portion and

5   exposing this polynucleotide to the chimeric

6   protein of step d) to yield a polynucleotide-

7   chimeric protein complex; and

8

9   f)   causing production of a non-sequence-specific

10   moiety able to bind to the non-protected portion

11   of the polynucleotide encoding the chimeric

12   protein to form a peptide display carrier package.

13

14   The present invention further provides a method of

15   screening a genetic library, said method comprising:

16

17   a)   exposing the polynucleotide members of said

18   library to multiple copies of a genetic construct

19   comprising a nucleotide sequence encoding a

20   nucleotide binding portion able to recognise and

21   bind to a specific sequence motif, under

22   conditions suitable for the polynucleotides of

23   said library each to be individually ligated into

24   one copy of said genetic construct, to create a

25   library of recombinant polynucleotides;

26

27   b)   exposing said recombinant polynucleotides to a

28   population of host cells, under conditions

29   suitable for transformation of said host cells by

30   said recombinant polynucleotides;

31

1   c)   selecting for transformed host cells;

2

3   d)   exposing said transformed host cells to conditions

4   suitable for expression of said recombinant

5   polynucleotide to yield a chimeric protein; and

6

7   e)   providing a recombinant polynucleotide comprising

8   the nucleotide sequence motif specifically

9   recognised by the nucleotide binding portion and

10   exposing this polynucleotide to the chimeric

11   protein of step d) to yield a polynucleotide-

12   chimeric protein complex;

13

14   f)   protecting any exposed portions of the

15   polynucleotide in the complex of step e) to form a

16   peptide display carrier package; and

17

18   g)   screening said peptide display carrier package to

19   select only those packages displaying a target

20   peptide portion having the characteristics

21   required.

22

23   Desirably in step a) the genetic construct is pDM12,

24   pDM14 or pDM16.

25

26   Desirably in step f) the peptide display package

27   carrier is extruded from the transformed host cell

28   without lysis of the host cell.

29

30   Generally the transformed host cells will be plated out

31   or otherwise divided into single colonies following

1 transformation and prior to expression of the chimeric

2 protein.

3

4 The screening step g) described above may look for a

5 particular target peptide either on the basis of

6 function (e.g. enzymic activity) or structure (e.g.

7 binding to a specific antibody). Once the peptide

8 display carrier package is observed to include a target

9 peptide with the desired characteristics, the

10 polynucleotide portion thereof (which of course encodes

11 the chimeric protein itself) can be amplified, cloned

12 and otherwise manipulated using standard genetic

13 engineering techniques.

14

15 The current invention differs from the prior art

16 teaching of the previous disclosures US Patent No

17 5,403,484 and US Patent No 5,571,698, as the invention

18 does not require outer surface transport signals, or

19 functional portions of viral coat proteins, to enable

20 the display of chimeric binding proteins on the outer

21 surface of the viral particle or genetic package.

22

23 The current invention also differs from the teaching of

24 WO-A-92/01047 and WO-A-92/20791, as no component of a

25 secreted replicable genetic display package, or viral

26 coat protein is required, to enable display of the

27 target peptide on the outer surface of the viral

28 particle.

29

30 The current invention differs from the teaching of US

31 Patent No 5498530, as it enables the display of

1 chimeric proteins, linked to the polynucleotide

2 encoding the chimeric protein, extra-cellularly, not in

3 the cytoplasm of a host cell. In the current invention

4 the chimeric proteins are presented on the outer

5 surface of a peptide display carrier package (PDCP)

6 which protects the DNA encoding the chimeric protein,

7 and does not require cell lysis to obtain access to the

8 chimeric protein-DNA complex. Finally, the current

9 invention does not rely upon the lacI DNA binding

10 protein to form the chimeric protein-DNA complex.

11

12 In one embodiment of the invention, the nucleotide

13 binding portion of the chimeric protein comprises a DNA

14 binding domain from one or more of the nuclear steroid

15 receptor family of proteins, or a functional equivalent

16 of such a domain. Particular examples include (but are

17 not limited to) a DNA binding domain of the [oestrogen]

18 <u>estrogen</u> receptor or the progesterone receptor, or

19 functional equivalents thereof. These domains can

20 recognise specific DNA sequences, termed hormone

21 response elements (HRE), which can be bound as both

22 double and single-stranded DNA. The DNA binding domain

23 of such nuclear steroid receptor proteins is preferred.

24

25 The [oestrogen] <u>estrogen</u> receptor is especially

26 referred to below by way of example, for convenience

27 since:

28 (a) The [oestrogen] <u>estrogen</u> receptor is a large

29 multifunctional polypeptide of 595 amino acids which

30 functions in the cytoplasm and nucleus of eukaryotic

31 cells (Green et al., 1986, Science 231: 1150-1154). A

1 minimal high affinity DNA binding domain (DBD) has been

2 defined between amino acids 176 and 282 (Mader et al.,

3 1993, Nucleic Acids Res. 21: 1125-1132). The

4 functioning of this domain (i.e. DNA binding) is not

5 inhibited by the presence of non-DNA binding domains at

6 both the N and C terminal ends of this domain, in the

7 full length protein.

8

9 (b) The [oestrogen] estrogen receptor DNA binding

10 domain fragment (amino acids 176-282) has been

11 expressed in *E. coli* and shown to bind to the specific

12 double stranded DNA [oestrogen] estrogen receptor

13 target HRE nucleotide sequence, as a dimer with a

14 similar affinity (0.5nM) to the parent molecule

15 (Murdoch et al. 1990, Biochemistry 29: 8377-8385; Mader

16 et al., 1993, Nucleic Acids Research 21: 1125-1132).

17 DBD dimerization on the surface of the PDCP should

18 result in two peptides displayed per particle. This

19 bivalent display can aid in the isolation of low

20 affinity peptides and peptides that are required to

21 form a bivalent conformation in order to bind to a

22 particular target, or activate a target receptor. The

23 [oestrogen] estrogen receptor is capable of binding to

24 its 38 base pair target HRE sequence, consensus

25 sequence:

26

27 1)   5'-TCAGGTCAGAGTGACCTGAGCTAAAATAACACATTCAG-3'

28   ("minus strand") SEQ ID No 77, and

29 2)   3'-AGTCCAGTCTCACTGGACTCGATTTTATTGTGTAAGTC-5'

30   ("plus strand") SEQ ID No 78,

31

1 with high affinity and specificity, under the salt and

2 pH conditions normally required for selection of

3 binding peptides. Moreover, binding affinity is

4 increased 60-fold for the single-stranded coding, or

5 "plus", strand (i.e. SEQ ID No 78) of the HRE

6 nucleotide sequence over the double stranded form of

7 the specific target nucleotide sequence (Peale et al.

8 1988, Proc. Natl. Acad. Sci. USA 85: 1038-1042;

9 Lannigan & Notides, 1989, Proc. Natl. Acad. Sci. USA

10 86: 863-867).

11

12 In an embodiment of the invention where the DNA binding

13 component of the peptide display carrier package is the

14 [oestrogen] estrogen receptor, the nucleotide (DNA)

15 binding portion contains a minimum sequence of amino

16 acids 176-282 of the [oestrogen] estrogen receptor

17 protein. In addition, the consensus [oestrogen]

18 estrogen receptor target HRE sequence is cloned in such

19 a way that if single stranded DNA can be produced then

20 the coding, or "plus", strand of the [oestrogen]

21 estrogen receptor HRE nucleotide sequence is

22 incorporated into single-stranded DNA. An example of a

23 vector suitable for this purpose is pUC119 (see Viera

24 et al., Methods in Enzymology, Vol 153, pages 3-11,

25 1987).

26

27 In a preferred embodiment of the invention a peptide

28 display carrier package (PDCP) can be assembled when a

29 bacterial host cell is transformed with a bacteriophage

30 vector, which vector comprises a recombinant

31 polynucleotide as described above. The expression

1   vector will also comprise the specific nucleotide motif

2   that can be bound by the nucleotide binding portion of

3   the chimeric protein. Expression of recombinant

4   polynucleotide results in the production of the

5   chimeric protein which comprises the target peptide and

6   the nucleotide binding portion. The host cell is grown

7   under conditions suitable for chimeric protein

8   expression and assembly of the bacteriophage particles,

9   and the association of the chimeric protein with the

10   specific nucleotide sequence in the expression vector.

11   In this embodiment, since the vector is a

12   bacteriophage, which replicates to produce a single-

13   stranded DNA, the nucleotide binding portion preferably

14   has an affinity for single-stranded DNA. Incorporation

15   of the vector single-stranded DNA-chimeric protein

16   complex into bacteriophage particles results in the

17   assembly of the peptide display carrier package (PDCP),

18   and display of the target peptide on the outer surface

19   of the PDCP.

20

21   In this embodiment both of the required elements for

22   producing peptide display carrier packages are

23   contained on the same vector. Incorporation of the DNA-

24   chimeric protein complex into a peptide display carrier

25   package (PDCP) is preferred as DNA degradation is

26   prevented, large numbers of PDCPs are produced per host

27   cell, and the PDCPs are easily separated from the host

28   cell without recourse to cell lysis.

29

30   In a more preferred embodiment, the vector of the is a

31   phagemid vector (for example pUC119) where expression

1   of the chimeric protein is controlled by an inducible

2   promoter. In this embodiment the PDCP can only be

3   assembled following infection of the host cell with

4   both phagemid vector and helper phage. The transfected

5   host cell is then cultivated under conditions suitable

6   for chimeric protein expression and assembly of the

7   bacteriophage particles.

8

9   In this embodiment the elements of the PDCP are

10   provided by two separate vectors. The phagemid derived

11   PDCP is superior to phagemid derived display packages

12   disclosed in WO-A-92/01047 where a proportion of

13   packages displaying bacteriophage coat protein fusion

14   proteins will contain the helper phage DNA, not the

15   fusion protein DNA sequence. In the current invention,

16   a PDCP can display the chimeric fusion protein only

17   when the package contains the specific nucleotide motif

18   recognised by the nucleotide binding portion. In most

19   embodiments this sequence will be present on the same

20   DNA segment that encodes the fusion protein. In

21   addition, the prior art acknowledges that when mutant

22   and wild type proteins are co-expressed in the same

23   bacterial cell, the wild type protein is produced

24   preferentially. Thus, when the wild type helper phage,

25   phage display system of WO-A-92/01047 is used, both

26   wild type gene pIII and target peptide-gene pIII

27   chimeric proteins are produced in the same cell. The

28   result of this is that the wild type gene pIII protein

29   is preferentially packaged into bacteriophage

30   particles, over the chimeric protein. In the current

1   invention, there is no competition with wild type

2   bacteriophage coat proteins for packaging.

3

4   Desirably the target peptide is displayed in a location

5   exposed to the external environment of the PDCP, after

6   the PDCP particle has been released from the host cell

7   without recourse to cell lysis. The target peptide is

8   then accessible for binding to its ligand. Thus, the

9   target peptide may be located at or near the N-terminus

10   or the C-terminus of a nucleotide binding domain, for

11   example the DNA binding domain of the [oestrogen]

12   estrogen receptor.

13

14   The present invention also provides a method for

15   screening a DNA library expressing one or more

16   polypeptide chains that are processed, folded and

17   assembled in the periplasmic space to achieve

18   biological activity. The PDCP may be assembled by the

19   following steps:

20

21   (a) Construction of N- or C-terminal DBD chimeric

22   protein fusions in a phagemid vector.

23   (i) When the target peptide is located at the N-

24   terminus of the nucleotide binding portion, a library

25   of DNA sequences each encoding a potential target

26   peptide is cloned into an appropriate location of an

27   expression vector (i.e. behind an appropriate promoter

28   and translation sequences and a sequence encoding a

29   signal peptide leader directing transport of the

30   downstream fusion protein to the periplasmic space) and

31   upstream of the sequence encoding the nucleotide

1   binding portion. In a preferred embodiment the DNA

2   sequence(s) of interest may be joined, by a region of

3   DNA encoding a flexible amino acid linker, to the 5'-

4   end of an [oestrogen] estrogen receptor DBD.

5   (ii) Alternatively, when the target peptide is

6   located at the C-terminus of the nucleotide binding

7   domain, a library of DNA sequences each encoding a

8   potential target peptide is cloned into the expression

9   vector so that the nucleotide sequence coding for the

10   nucleotide binding portion is upstream of the cloned

11   DNA target peptide encoding sequences, said nucleotide

12   binding portion being positioned behind an appropriate

13   promoter and translation sequences and a sequence

14   encoding a signal peptide leader directing transport of

15   the downstream fusion protein to the periplasmic space.

16   In a preferred embodiment, DNA sequence(s) of interest

17   may be joined, by a region of DNA encoding a flexible

18   amino acid linker [oestrogen] estrogen receptor DBD DNA

19   sequence.

20

21   Located on the expression vector is the specific HRE

22   nucleotide sequence recognised, and bound, by the

23   [oestrogen] estrogen receptor DBD. In order to vary the

24   number of chimeric proteins displayed on each PDCP

25   particle, this sequence can be present as one or more

26   copies in the vector.

27

28   (b) Incorporation into the PDCP. Non-lytic helper

29   bacteriophage infects host cells containing the

30   expression vector. Preferred types of bacteriophage

31   include the filamentous phage fd, fl and M13. In a

1   more preferred embodiment the bacteriophage may be

2   M13K07.

3

4   The protein(s) of interest are expressed and

5   transported to the periplasmic space, and the properly

6   assembled proteins are incorporated into the PDCP

7   particle by virtue of the high affinity interaction of

8   the DBD with the specific target nucleotide sequence

9   present on the phagemid vector DNA which is naturally

10   packaged into phage particles in a single-stranded

11   form. The high affinity interaction between the DBD

12   protein and its specific target nucleotide sequence

13   prevents displacement by bacteriophage coat proteins

14   resulting in the incorporation of the protein(s) of

15   interest onto the surface of the PDCP as it is extruded

16   from the cell.

17

18   (c) Selection of the peptide of interest. Particles

19   which display the peptide of interest are then selected

20   from the culture by affinity enrichment techniques.

21   This is accomplished by means of a ligand specific for

22   the protein of interest, such as an antigen if the

23   protein of interest is an antibody. The ligand may be

24   presented on a solid surface such as the surface of an

25   ELISA plate, or in solution. Repeating the affinity

26   selection procedure provides an enrichment of clones

27   encoding the desired sequences, which may then be

28   isolated for sequencing, further cloning and/or

29   expression.

30

1   Numerous types of libraries of peptides fused to the

2   DBD can be screened under this embodiment including:

3

4   (i) Random peptide sequences encoded by synthetic

5   DNA of variable length.

6

7   (ii) Single-chain Fv antibody fragments. These

8   consist of the antibody heavy and light chain

9   variable region domains joined by a flexible

10   linker peptide to create a single-chain antigen

11   binding molecule.

12

13   (iii) Random fragments of naturally occurring

14   proteins isolated from a cell population

15   containing an activity of interest.

16

17   In another embodiment the invention concerns methods

18   for screening a DNA library whose members require more

19   than one chain for activity, as required by, for

20   example, antibody Fab fragments for ligand binding. In

21   this embodiment heavy or light chain antibody DNA is

22   joined to a nucleotide sequence encoding a DNA binding

23   domain of, for example, the [oestrogen] estrogen

24   receptor in a phagemid vector. Typically the antibody

25   DNA library sequences for either the heavy (VH and CH1)

26   or light chain (VL and CL) genes are inserted in the 5'

27   region of the [oestrogen] estrogen receptor DBD DNA,

28   behind an appropriate promoter and translation

29   sequences and a sequence encoding a signal peptide

30   leader directing transport of the downstream fusion

31   protein to the periplasmic space.

1

2 Thus, a DBD fused to a DNA library member-encoded

3 protein is produced and assembled in to the viral

4 particle after infection with bacteriophage. The second

5 and any subsequent chain(s) are expressed separately

6 either:

7

8 (a) from the same phagemid vector containing the DBD

9 and the first polypeptide fusion protein,

10 or

11

12 (b) from a separate region of DNA which may be present

13 in the host cell nucleus, or on a plasmid, phagemid or

14 bacteriophage expression vector that can co-exist, in

15 the same host cell, with the first expression vector,

16 so as to be transported to the periplasm where they

17 assemble with the first chain that is fused to the DBD

18 protein as it exits the cell. Peptide display carrier

19 packages (PDCP) which encode the protein of interest

20 can then be selected by means of a ligand specific for

21 the protein.

22

23 In yet another embodiment, the invention concerns

24 screening libraries of bi-functional peptide display

25 carrier packages where two or more activities of

26 interest are displayed on each PDCP. In this

27 embodiment, a first DNA library sequence(s) is inserted

28 next to a first DNA binding domain (DBD) DNA sequence,

29 for example the [oestrogen] estrogen receptor DBD, in

30 an appropriate vector, behind an appropriate promoter

31 and translation sequences and a sequence encoding a

1 signal peptide leader directing transport of this first
2 chimeric protein to the periplasmic space. A second
3 chimeric protein is also produced from the same, or
4 separate, vector by inserting a second DNA library
5 sequence(s) next to a second DBD DNA sequence which is
6 different from the first DBD DNA sequence, for example
7 the progesterone receptor DBD, behind an appropriate
8 promoter and translation sequences and a sequence
9 encoding a signal peptide leader. The first, or only,
10 vector contains the specific HRE nucleotide sequences
11 for both [oestrogen] estrogen and progesterone
12 receptors. Expression of the two chimeric proteins,
13 results in a PDCP with two different chimeric proteins
14 displayed. As an example, one chimeric protein could
15 possess a binding activity for a particular ligand of
16 interest, while the second chimeric protein could
17 possess an enzymatic activity. Binding by the PDCP to
18 the ligand of the first chimeric protein could then be
19 detected by subsequent incubation with an appropriate
20 substrate for the second chimeric protein. In an
21 alternative embodiment a bi-functional PDCP may be
22 created using a single DBD, by cloning one peptide at
23 the 5'-end of the DBD, and a second peptide at the 3'-
24 end of the DBD. Expression of this single bi-functional
25 chimeric protein results in a PDCP with two different
26 activities.
27
28 We have investigated the possibility of screening
29 libraries of peptides, fused to a DNA binding domain
30 and displayed on the surface of a display package, for
31 particular peptides with a biological activity of

1   interest and recovering the DNA encoding that activity.

2   Surprisingly, by manipulating the [oestrogen] <u>estrogen</u>

3   receptor DNA binding domain in conjunction with M13

4   bacteriophage we have been able to construct novel

5   particles which display large biologically functional

6   molecules, that allows enrichment of particles with the

7   desired specificity.

8

9   The invention described herein provides a significant

10   breakthrough in DNA library screening technology.

11

12   The invention will now be further described by

13   reference to the non-limiting examples and figures

14   below.

15

16   **Description of Figures**

17

18   Figure 1 shows the pDM12 N-terminal fusion [oestrogen]

19   <u>estrogen</u> receptor DNA binding domain expression vector

20   nucleotide sequence (SEQ ID No 1), between the HindIII

21   and EcoRI restriction sites, comprising a pelB leader

22   secretion sequence (in italics) (SEQ ID No 2), multiple

23   cloning site containing SfiI and NotI sites, flexible

24    $(glycine)_4$-serine linker sequence (boxed), a fragment of

25   the [oestrogen] <u>estrogen</u> receptor gene comprising amino

26   acids 176-282 (SEQ ID No 3) of the full length

27   molecule, and the 38 base pair consensus [oestrogen]

28   <u>estrogen</u> receptor DNA binding domain HRE sequence.

29

30   Figure 2 shows the OD_{450nm} ELISA data for negative

31   control M13K07 phage, and single-clone PDCP display

1 culture supernatants (#1-4, see Example 3) isolated by

2 selection of the lymphocyte cDNA-pDM12 library against

3 anti-human immunoglobulin kappa antibody.

4

5 Figure 3 shows partial DNA (SEQ ID No 4) and amino acid

6 (SEQ ID No 5) sequence for the human immunoglobulin

7 kappa constant region (Kabat, E. A. et al., Sequences

8 of Proteins of Immunological Interest. 4th edition. U.S.

9 Department of Health and Human Services. 1987), and

10 ELISA positive clones #2 (SEQ ID Nos 6 and 7) and #3

11 (SEQ ID Nos 8 and 9) from Figure 2 which confirms the

12 presence of human kappa constant region DNA in-frame

13 with the pelB leader sequence (pelB leader sequence is

14 underlined, the leader sequence cleavage site is

15 indicated by an arrow). The differences in the 5'-end

16 sequence demonstrates that these two clones were

17 selected independently from the library stock. The PCR

18 primer sequence is indicated in bold, clone #2 was

19 originally amplified with CDNAPCRBAK1 and clone #3 was

20 amplified with CDNAPCRBAK2.

21

22 Figure 4 shows the pDM14 N-terminal fusion [oestrogen]

23 estrogen receptor DNA binding domain expression vector

24 nucleotide sequence (SEQ ID No 10), between the HindIII

25 and EcoRI restriction sites, comprising a pelB leader

26 secretion sequence (in italics)(SEQ ID No 11), multiple

27 cloning site containing SfiI and NotI sites, flexible

28 (glycine)$_4$-serine linker sequence (boxed), a fragment of

29 the [oestrogen] estrogen receptor gene comprising amino

30 acids 176-282 (see SEQ ID No 12) of the full length

31 molecule, and the two 38 base pair [oestrogen] estrogen

1   receptor DNA binding domain HRE sequences (HRE 1 and

2   HRE 2).

3

4   Figure 5 shows the pDM16 C-terminal fusion [oestrogen]

5   <u>estrogen</u> receptor DNA binding domain expression vector

6   nucleotide sequence (SEQ ID No 13), between the HindIII

7   and EcoRI restriction sites, comprising a pelB leader

8   secretion sequence (in italics), a fragment of the

9   [oestrogen] <u>estrogen</u> receptor gene comprising amino

10 acids 176-282 (SEQ ID No 14) of the full length

11 molecule, flexible (glycine)$_4$-serine linker sequence

12 (boxed), multiple cloning site containing SfiI and NotI

13 sites and the 38 base pair [oestrogen] <u>estrogen</u>

14 receptor DNA binding domain HRE sequence.

15

16 Figure 6 shows the OD$_{450nm}$ ELISA data for N-cadherin-

17 pDM16 C-terminal display PDCP binding to anti-pan-

18 cadherin monoclonal antibody in serial dilution ELISA

19 as ampicillin resitance units (a.r.u.). Background

20 binding of negative control M13K07 helper phage is also

21 shown.

22

23 Figure 7 shows the OD$_{450nm}$ ELISA data for *in vivo*

24 biotinylated PCC-pDM16 C-terminal display PDCP binding

25 to streptavidin in serial dilution ELISA as ampicillin

26 resitance units (a.r.u.). Background binding of

27 negative control M13K07 helper phage is also shown.

28

29 Figure 8 shows the OD$_{450nm}$ ELISA data for a human scFv

30 PDCP isolated from a human scFv PDCP display library

31 selected against substance P. The PDCP was tested

1 against streptavidin (1), streptavidin-biotinylated

2 substance P (2), and streptavidin-biotinylated CGRP

3 (3), in the presence (B) or absence (A) of free

4 substance P.

5

6 Figure 9 shows the DNA (SEQ ID Nos 15 and 17) and amino

7 acid (SEQ ID No 16 and 18) sequence of the substance P

8 binding scFv isolated from a human scFv PDCP display

9 library selected against substance P. Heavy chain (SEQ

10 ID Nos 15 and 16) and light chain (SEQ ID Nos 17 and

11 18) variable region sequence is shown with the CDRs

12 underlined and highlighted in bold.

13

14 **Materials and Methods**

15 The following procedures used by the present applicant

16 are described in Sambrook, J., et al., 1989 supra.:

17 restriction enzyme digestion, ligation, preparation of

18 electrocompetent cells, electroporation, analysis of

19 restriction enzyme digestion products on agarose gels,

20 DNA purification using phenol/chloroform, preparation

21 of 2xTY medium and plates, preparation of ampicillin,

22 kanamycin, IPTG (Isopropyl β-D-Thiogalactopyranoside)

23 stock solutions, and preparation of phosphate buffered

24 saline.

25

26 Restriction enzymes, T4 DNA ligase and cDNA synthesis

27 reagents (Superscript plasmid cDNA synthesis kit) were

28 purchased from Life Technologies Ltd (Paisley,

29 Scotland, U.K.). Oligonucleotides were obtained from

30 Cruachem Ltd (Glasgow, Scotland, U.K.), or Genosys

31 Biotechnologies Ltd (Cambridge, U.K.). Taq DNA

1 polymerase, Wizard SV plasmid DNA isolation kits,

2 streptavidin coated magnetic beads and mRNA isolation

3 reagents (PolyATract 1000) were obtained from Promega

4 Ltd (Southampton, Hampshire, U.K.). Taqplus DNA

5 polymerase was obtained from Stratagene Ltd (Cambridge,

6 U.K.). PBS, BSA, streptavidin, substance P and anti-pan

7 cadherin antibody were obtained from SIGMA Ltd (Poole,

8 Dorset, U.K.). Anti-M13-HRP conjugated antibody,

9 Kanamycin resistant M13K07 helper bacteriophage and

10 RNAguard were obtained from Pharmacia Ltd (St. Albans,

11 Herts, U.K.) and anti-human Igκ antibody from Harlan-

12 Seralab (Loughborough, Leicestershire, U.K.)

13 Biotinylated substance P and biotinylated calcitonin

14 gene related peptide (CGRP) were obtained from

15 Peninsula Laboratories (St. Helens, Merseyside, U.K.).

16

17 Specific embodiments of the invention are given below

18 in Examples 1 to 9.

19

1 **Example 1. Construction of a N-terminal PDCP display**

2 **phagemid vector pDM12.**

3

4 The pDM12 vector was constructed by inserting an

5 [oestrogen] <u>estrogen</u> receptor DNA binding domain,

6 modified by appropriate PCR primers, into a phagemid

7 vector pDM6. The pDM6 vector is based on the pUC119

8 derived phage display vector pHEN1 (Hoogenboom et al.,

9 1991, Nucleic Acids Res. 19: 4133-4137). It contains

10 (Gly)$_4$Ser linker, Factor Xa cleavage site, a full length

11 gene III, and streptavidin tag peptide sequence

12 (Schmidt, T.G. and Skerra, A., 1993, Protein

13 Engineering 6: 109-122), all of which can be removed by

14 NotI-EcoRI digestion and agarose gel electrophoresis,

15 leaving a pelB leader sequence, SfiI, NcoI and PstI

16 restriction sites upstream of the digested NotI site.

17 The cloned DNA binding domain is under the control of

18 the lac promoter found in pUC119.

19

20 **Preparation of pDM6**

21

22 The pDM12 vector was constructed by inserting an

23 [oestrogen] <u>estrogen</u> receptor DNA binding domain,

24 modified by appropriate PCR primers, into a phagemid

25 vector pDM6. The pDM6 vector is based on the gene pIII

26 phage display vector pHEN1 (Hoogenboom et al., 1991,

27 Nucleic Acids Res. 19: 4133-4137), itself derived from

28 pUC119 (Viera, J. and Messing, J., 1987, Methods in

29 Enzymol. 153: 3-11). It was constructed by amplifying

30 the pIII gene in pHEN1 with two oligonucleotides:

31

1 PDM6BAK: 5 -TTT TCT GCA GTA ATA GGC GGC CGC AGG GGG AGG

2 AGG GTC CAT CGA AGG TCG CGA AGC AGA GAC TGT TGA AAG T-3

3 (SEQ ID No 19) and

4

5 PDM6FOR: 5 - TTT TGA ATT CTT ATT AAC CAC CGA ACT GCG

6 GGT GAC GCC AAG CGC TTG CGG CCG TTA AGA CTC CTT ATT ACG

7 CAG-3 (SEQ ID No 20).

8

9 and cloning the PstI-EcoRI digested PCR product back

10 into similarly digested pHEN1, thereby removing the

11 c-myc tag sequence and supE TAG codon from pHEN1. The

12 pDM6 vector contains a (Gly)$_4$Ser linker, Factor Xa

13 cleavage site, a full length gene III, and streptavidin

14 tag peptide sequence (Schmidt, T.G. and Skerra, A.,

15 1993, Protein Engineering 6: 109-122), all of which can

16 be removed by NotI-EcoRI digestion and agarose gel

17 electrophoresis, leaving a pelB leader sequence, SfiI,

18 NcoI and PstI restriction sites upstream of the

19 digested NotI site. The cloned DNA binding domain is

20 under the control of the lac promoter found in pUC119.

21

22 The [oestrogen] estrogen receptor DNA binding domain

23 was isolated from cDNA prepared from human bone marrow

24 (Clontech, Palo Alto, California, U.S.A.). cDNA can be

25 prepared by many procedures well known to those skilled

26 in the art. As an example, the following method using a

27 Superscript plasmid cDNA synthesis kit can be used:

28

29 **(a) First strand synthesis**.

30

1   5µg of bone marrow mRNA, in 5µl DEPC-treated water was

2   thawed on ice and 2µl (50pmol) of cDNA synthesis primer

3   (5'-AAAAGCGGCCGCACTGGCCTGAGAGA(N)$_6$-3') (SEQ ID No 21)

4   was added to the mRNA and the mixture heated to 70°C for

5   10 minutes, then snap-chilled on ice and spun briefly

6   to collect the contents to the bottom of the tube. The

7   following were then added to the tube:

8   1000u/ml RNAguard   1µl

9   5x first strand buffer   4µl

10   0.1M DTT   2µl

11   10mM dNTPs   1µl

12   200u/µl SuperScript II reverse transcriptase 5µl

13   The mixture was mixed by pipetting gently and incubated

14   at 37°C for 1 hour, then placed on ice.

15

16   **(b) Second strand synthesis.**

17

18   The following reagents were added to the first strand

19   reaction:

20   DEPC-treated water   93µl

21   5x second strand buffer   30µl

22   10mM dNTPs   3µl

23   10u/µl *E. coli* DNA ligase   1µl

24   10u/µl *E. coli* DNA polymerase   4µl

25   2u/µl *E. coli* RNase H   1µl

26   The reaction was vortex mixed and incubated at 16°C for

27   2 hours. 2µl (10u) of T4 DNA polymerase was added and

28   incubation continued at 16°C for 5 minutes. The reaction

29   was placed on ice and 10µl 0.5M EDTA added, then

30   phenol-chloroform extracted, precipitated and vacuum

31   dried.

(c) Sal I adaptor ligation.

The cDNA pellet was resuspended in 25µl DEPC-treated
water, and ligation set up as follows.

cDNA	25µl
5x T4 DNA ligase buffer	10µl
1µg/µl *Sal* I adapters*	10µl
1u/µlT4 DNA ligase	5µl

*Sal I adapters: TCGACCCACGCGTCCG-3' (SEQ ID No 22)
                     GGGTGCCGAGGC-5' (SEQ ID No 23)

The ligation was mixed gently and incubated for 16
hours at 16°C, then phenol-chloroform extracted,
precipitated and vacuum dried. The cDNA/adaptor pellet
was resuspended in 41µl of DEPC-treated water and
digested with 60 units of NotI at 37°C for 2 hours, then
phenol-chloroform extracted, precipitated and vacuum
dried. The cDNA pellet was re-dissolved in 100µl TEN
buffer (10mM Tris pH 7.5, 0.1mM EDTA, 25mM NaCl) and
size fractionated using a Sephacryl S-500 HR column to
remove unligated adapters and small cDNA fragments
(<400bp) according to the manufacturers instructions.
Fractions were checked by agarose gel electrophoresis
and fractions containing cDNA less than 400 base pairs
discarded, while the remaining fractions were pooled.

**(d) PCR amplification of [oestrogen] estrogen receptor
DNA binding domain.**

The [oestrogen] estrogen receptor was PCR amplified
from 5µl (150-250ng) of bone marrow cDNA using 25pmol

1 of each of the primers pDM12FOR (SEQ ID No 24) (5'-

2 AAAAGAATTCTGAATGTGTTATTTTAGCTCAGGTCACTCTGACCTGATTATCAAG

3 ACCCCACTTCACCCCCT) and pDM12BAK (SEQ ID No 25) (5'-

4 AAAAGCGGCCGCAGGGGGAGGAGGGTCCATGGAATCTGCCAAGGAG-3') in

5 two 50µl reactions containing 0.1mM dNTPs, 2.5 units

6 Taq DNA polymerase, and 1x PCR reaction buffer (10mM

7 Tris-HCl pH 9.0, 5mM KCl, 0.01% Triton X®-100, 1.5mM

8 MgCl$_2$) (Promega Ltd, Southampton, U.K.). The pDM12FOR

9 primer anneals to the 3'-end of the DNA binding domain

10 of the [oestrogen] estrogen receptor and incorporates

11 two stop codons, the 38 base pair consensus [oestrogen]

12 estrogen receptor HRE sequence, and an EcoRI

13 restriction site. The pDM12BAK primer anneals to the

14 5'-end of the DNA binding domain of the [oestrogen]

15 estrogen receptor and incorporates the (Gly)$_4$Ser linker

16 and the NotI restriction site.

17

18 Reactions were overlaid with mineral oil and PCR

19 carried out on a Techne PHC-3 thermal cycler for 30

20 cycles of 94°C, 1 minute; 65°C, 1 minute; 72°C, 1

21 minute. Reaction products were electrophoresed on an

22 agarose gel, excised and products purified from the gel

23 using a Geneclean II kit according to the manufacturers

24 instructions (Bio101, La Jolla, California, U.S.A.).

25

26 **(e) Restriction digestion and ligation.**

27

28 The PCR reaction appended NotI and EcoRI restriction

29 sites, the (Gly)$_4$Ser linker, stop codons and the 38 base

30 pair [oestrogen] estrogen receptor target HRE

31 nucleotide sequence to the [oestrogen] estrogen

1 receptor DNA binding domain sequence (see Figure 1).

2 The DNA PCR fragment and the target pDM6 vector

3 (approximately 500ng) were NotI and EcoRI digested for

4 1 hour at 37°C, and DNA purified by agarose gel

5 electrophoresis and extraction with Geneclean II kit

6 (Bio101, La Jolla, California, U.S.A.). The [oestrogen]

7 estrogen receptor DNA binding domain cassette was

8 ligated into the NotI-EcoRI digested pDM6 vector

9 overnight at 16°C, phenol/chloroform extracted and

10 precipitated then electroporated into TG1 *E. coli*

11 (genotype: K12, (Δlac-pro), supE, thi, hsD5/F'traD36,

12 proA⁺B⁺, LacI�q, LacZΔ15) and plated onto 2xTY agar

13 plates supplemented with 1% glucose and 100μg/ml

14 ampicillin. Colonies were allowed to grow overnight at

15 37°C. Individual colonies were picked into 5ml 2xTY

16 supplemented with 1% glucose and 100μg/ml ampicillin

17 and grown overnight at 37°C. Double stranded phagemid

18 DNA was isolated with a Wizard SV plasmid DNA isolation

19 kit and the sequence confirmed with a Prism dyedeoxy

20 cycle sequencing kit (Perkin-Elmer, Warrington,

21 Lancashire, U.K.) using M13FOR (SEQ ID No 26) (5'-

22 GTAAAACGACGGCCAGT) and M13REV (SEQ ID No 27) (5'-

23 GGATAACAATTTCACACAGG) oligonucleotides. The pDM12 PDCP

24 display vector DNA sequence between the HindIII and

25 EcoRI restriction sites is shown in Figure 1.

26

27 **Example 2. Insertion of a random-primed human**

28 **lymphocyte cDNA into pDM12 and preparation of a master**

29 **PDCP stock.**

30

1   Libraries of peptides can be constructed by many

2   methods known to those skilled in the art. The example

3   given describes a method for constructing a peptide

4   library from randomly primed cDNA, prepared from mRNA

5   isolated from a partially purified cell population.

6

7   mRNA was isolated from approximately 10^9 human

8   peripheral blood lymphocytes using a polyATract 1000

9   mRNA isolation kit (Promega, Southampton, UK). The cell

10   pellet was resuspended in 4ml extraction buffer (4M

11   guanidine thiocyanate, 25mM sodium citrate pH 7.1, 2%

12   β-mercaptoethanol). 8ml of pre-heated (70°C) dilution

13   buffer (6xSSC, 10mM Tris pH 7.4, 1mM EDTA, 0.25% SDS,

14   1% β-mercaptoethanol) was added to the homogenate and

15   mixed thoroughly by inversion. 10μl of biotinylated

16   oligo-dT (50 pmol/μl) was added, mixed and the mixture

17   incubated at 70°C for 5 minutes. The lymphocyte cell

18   lysate was transferred to 6x 2ml sterile tubes and spun

19   at 13,000 rpm in a microcentrifuge for ten minutes at

20   ambient temperature to produce a cleared lysate. During

21   this centrifugation, streptavidin coated magnetic beads

22   were resuspended and 6ml transferred to a sterile 50ml

23   Falcon tube, then placed in the magnetic stand in a

24   horizontal position until all the beads were captured.

25   The supernatant was carefully poured off and beads

26   resuspended in 6ml 0.5xSSC, then the capture repeated.

27   This wash was repeated 3 times, and beads resuspended

28   in a final volume of 6ml 0.5xSSC. The cleared lysate

29   was added to the washed beads, mixed by inversion and

30   incubated at ambient temperature for 2 minutes, then

31   beads captured in the magnetic stand in a horizontal

1   position. The beads were resuspended gently in 2ml

2   0.5xSSC and transferred to a sterile 2ml screwtop tube,

3   then captured again in the vertical position, and the

4   wash solution discarded. This wash was repeated twice

5   more. 1ml of DEPC-treated water was added to the beads

6   and mixed gently. The beads were again captured and the

7   eluted mRNA transferred to a sterile tube. 50µl was

8   electrophoresed to check the quality and quantity of

9   mRNA, while the remainder was precipitated with 0.1

10   volumes 3M sodium acetate and three volumes absolute

11   ethanol at -80°C overnight in 4 aliquots in sterile

12   1.5ml screwtop tubes.

13

14   Double stranded cDNA was synthesised as described in

15   Example 1 using 5µg of lymphocyte mRNA as template.

16   The cDNA was PCR amplified using oligonucleotides

17   CDNAPCRFOR (SEQ ID No 28) (5'-

18   AAAGCGGCCGCACTGGCCTGAGAGA), which anneals to the cDNA

19   synthesis oligonucleotide described in Example 1 which

20   is present at the 3'-end of all synthesised cDNA

21   molecules incorporates a NotI restriction site, and an

22   equimolar mixture of CDNAPCRBAK1, CDNAPCRBAK2 and

23   CDNAPCRBAK3.

24   CDNAPCRBAK1: (SEQ ID No 29) 5'-

25   AAAAGGCCCAGCCGGCCATGGCCCAGCCCACCACGCGTCCG,

26   CDNAPCRBAK2: (SEQ ID No 30) 5'-

27   AAAAGGCCCAGCCGGCCATGGCCCAGTCCCACCACGCGTCCG,

28   CDNAPCRBAK3: (SEQ ID No 31) 5'-

29   AAAAGGCCCAGCCGGCCATGGCCCAGTACCCACCACGCGTCCG),

30   all three of which anneal to the SalI adaptor sequence

31   found at the 5'-end of the cDNA and incorporate a SfiI

1 restriction site at the cDNA 5'-end. Ten PCR reactions

2 were carried out using 2µl of cDNA (50ng) per reaction

3 as described in Example 1 using 25 cycles of 94°C, 1

4 minute; 60°C, 1 minute; 72°C, 2 minutes. The reactions

5 were pooled and a 20µl aliquot checked by agarose gel

6 electrophoresis, the remainder was phenol/chloroform

7 extracted and ethanol precipitated and resuspended in

8 100µl sterile water. 5µg of pDM12 vector DNA and

9 lymphocyte cDNA PCR product were SfiI-NotI digested

10 phenol/chloroform extracted and small DNA fragments

11 removed by size selection on Chromaspin 1000 spin

12 columns (Clontech, Palo Alto, California, U.S.A.) by

13 centrifugation at 700g for 2 minutes at room

14 temperature. Digested pDM12 and lymphocyte cDNA were

15 ethanol precipitated and ligated together for 16 hours

16 at 16°C. The ligated DNA was precipitated and

17 electroporated in to TG1 *E. coli*. Cells were grown in

18 1ml SOC medium per cuvette used for 1 hour at 37°C, and

19 plated onto 2xTY agar plates supplemented with 1%

20 glucose and 100µg/ml ampicillin. 10^{-4}, 10^{-5} and 10^{-6}

21 dilutions of the electroporated bacteria were also

22 plated to assess library size. Colonies were allowed to

23 grow overnight at 30°C. $2x10^8$ ampicillin resistant

24 colonies were recovered on the agar plates.

25 The bacteria were then scraped off the plates into 40ml

26 2xTY broth supplemented with 20% glycerol, 1% glucose

27 and 100µg/ml ampicillin. 5ml was added to a 20ml 2xTY

28 culture broth supplemented with 1% glucose and 100µg/ml

29 ampicillin and infected with 10^{11} kanamycin resistance

30 units (kru) M13K07 helper phage at 37°C for 30 minutes

1 without shaking, then for 30 minutes with shaking at

2 200rpm. Infected bacteria were transferred to 200ml

3 2xTY broth supplemented with 25µg/ml kanamycin,

4 100µg/ml ampicillin, and 20µM IPTG, then incubated

5 overnight at 37°C, shaking at 200rpm. Bacteria were

6 pelleted at 4000rpm for 20 minutes in 50ml Falcon

7 tubes, and 40ml 2.5M NaCl/20% PEG 6000 was added to

8 200ml of particle supernatant, mixed vigorously and

9 incubated on ice for 1 hour to precipitate PDCP

10 particles. Particles were pelleted at 11000rpm for 30

11 minutes in 250ml Oakridge tubes at 4°C in a Sorvall RC5B

12 centrifuge, then resuspended in 2ml PBS buffer after

13 removing all traces of PEG/NaCl with a pipette, then

14 bacterial debris removed by a 5 minute 13500rpm spin in

15 a microcentrifuge. The supernatent was filtered through

16 a 0.45µm polysulfone syringe filter and stored at -20°C.

17

18 **Example 3. Isolation of human immunoglobulin kappa**

19 **light chains by repeated rounds of selection against**

20 **anti-human kappa antibody.**

21

22 For the first round of library selection a 70x11mm NUNC

23 Maxisorp Immunotube (Life Technologies, Paisley,

24 Scotland U.K.) was coated with 2.5ml of 10µg/ml of

25 anti-human kappa antibody (Seralab, Crawley Down,

26 Sussex, U.K.) in PBS for 2 hours at 37°C. The tube was

27 rinsed three times with PBS (fill & empty) and blocked

28 with 3ml PBS/2% BSA for 2 hours at 37°C and washed as

29 before. 4×10^{12} a.r.u. of pDM12-lymphocyte cDNA PDCP

30 stock was added in 2ml 2% BSA/PBS/0.05% Tween 20, and

1 incubated for 30 minutes on a blood mixer, then for 90

2 minutes standing at ambient temperature. The tube was

3 washed ten times with PBS/0.1% Tween 20, then a further

4 ten times with PBS only. Bound particles were eluted in

5 1ml of freshly prepared 0.1M triethylamine for 10

6 minutes at ambient temperature on a blood mixer. Eluted

7 particles were transferred to 0.5ml 1M Tris pH 7.4,

8 vortex mixed briefly and transferred to ice.

9

10 Neutralised particles were added to 10ml log phase TG1

11 E coli bacteria (optical density: OD_{600nm} 0.3-0.5) and

12 incubated at 37°C without shaking for 30 minutes, then

13 with shaking at 200rpm for 30 minutes. 10^{-3}, 10^{-4} & 10^{-5}

14 dilutions of the infected culture were prepared to

15 estimate the number of particles recovered, and the

16 remainder was spun at 4000 rpm for 10 minutes, and the

17 pellet resuspended in 300μl 2xTY medium by vortex

18 mixing. Bacteria were plated onto 2xTY agar plates

19 supplemented with 1% glucose and 100μg/ml ampicillin.

20 Colonies were allowed to grow overnight at 30°C.

21

22 A PDCP stock was prepared from the bacteria recovered

23 from the first round of selection, as described in

24 Example 2 from a 100ml overnight culture. 250μl of the

25 round 1 amplified PDCP stock was then selected against

26 anti-human kappa antibody as described above with the

27 tube was washed twelve times with PBS/0.1% Tween 20,

28 then a further twelve times with PBS only.

29

30 To identify selected clones, eighty-eight individual

31 clones recovered from the second round of selection

1   were then tested by ELISA for binding to anti-human

2   kappa antibody. Individual colonies were picked into

3   100µl 2xTY supplemented with 100µg/ml ampicillin and 1%

4   glucose in 96-well plates (Costar) and incubated at 37°C

5   and shaken at 200rpm for 4 hours. 25µl of each culture

6   was transferred to a fresh 96-well plate, containing

7   25µl/well of the same medium plus 10^7 k.r.u. M13K07

8   kanamycin resistant helper phage and incubated at 37°C

9   for 30 minutes without shaking, then incubated at 37°C

10   and shaken at 200rpm for a further 30 minutes. 160µl of

11   2xTY supplemented with 100µg/ml ampicillin, 25µg/ml

12   kanamycin, and 20µM IPTG was added to each well and

13   particle amplification continued for 16 hours at 37°C

14   while shaking at 200rpm. Bacterial cultures were spun

15   in microtitre plate carriers at 2000g for 10 minutes at

16   4°C in a benchtop centrifuge to pellet bacteria and

17   culture supernatant used for ELISA.

18

19   A Dynatech Immulon 4 ELISA plate was coated with

20   200ng/well anti-human kappa antibody in 100µl /well PBS

21   for one hour at 37°C. The plate was washed 2x200µl/well

22   PBS and blocked for 1 hour at 37°C with 200µl/well 2%

23   BSA/PBS and then washed 2x200µl/well PBS. 50µl PDCP

24   culture supernatant was added to each well containing

25   50µl/well 4% BSA/PBS/0.1%Tween 20, and allowed to bind

26   for 1 hour at ambient temperature. The plate was washed

27   three times with 200µl/well PBS/0.1% Tween 20, then

28   three times with 200µl/well PBS. Bound PDCPs were

29   detected with 100µl/well, 1:5000 diluted anti-M13-HRP

30   conjugate (Pharmacia) in 2% BSA/PBS/0.05% Tween 20 for

1   1 hour at ambient temperature and the plate washed six

2   times as above. The plate was developed for 5 minutes

3   at ambient temperature with 100µl/well freshly prepared

4   TMB (3,3',5,5'-Tetramethylbenzidine) substrate buffer

5   (0.005% H_2O_2, 0.1mg/ml TMB in 24mM citric acid/52mM

6   sodium phosphate buffer pH 5.2). The reaction was

7   stopped with 100µl/well 12.5% H_2SO_4 and read at 450nm.

8   (ELISA data for binding clones is shown in Figure 2).

9

10   These clones were then sequenced with M13REV primer

11   (SEQ ID No 27) as in Example 1. The sequence of two of

12   the clones isolated is shown in Figure 3 (see SEQ ID

13   Nos 7 to 10).

14

15   **Example 4. Construction of the pDM14 N-terminal display**

16   **vector**

17

18   It would be useful to design vectors that contain a

19   second DBD binding sequence, such as a second

20   [oestrogen] estrogen receptor HRE sequence, thus

21   allowing the display of increased numbers of peptides

22   per PDCP. Peale et al. (1988, Proc. Natl. Acad. Sci.

23   USA 85: 1038-1042) describe a number of [oestrogen]

24   estrogen receptor HRE sequences. These sequences were

25   used to define an HRE sequence, which differs from that

26   cloned in pDM12, which we used to create a second N-

27   terminal display vector (pDM14).

28   The oligonucleotide: 5'-AAAAGAATTCGAGGTTACATTAACTTTGTT

29   CCGGTCAGACTGACCCAAGTCGACCTGAATGTGTTATTTTAG-3' (SEQ ID

30   No 32) was synthesised and used to mutagenise pDM12 by

31   PCR with pDM12BAK oligonucleotide as described in

1   Example 1 using 100ng pDM12 vector DNA as template. The

2   resulting DNA fragment, which contained the [oestrogen]

3   <u>estrogen</u> receptor DBD and two HRE sequences separated

4   by a SalI restriction enzyme site, was NotI-EcoRI

5   restriction enzyme digested and cloned into NotI-EcoRI

6   digested pDM12 vector DNA as described in Example 1 to

7   create pDM14. The sequence of pDM14 between the HindIII

8   and EcoRI restriction enzyme sites was checked by DNA

9   sequencing. The final vector sequence between these two

10 sites is shown in Figure 4 (see SEQ ID Nos 11 and 12).

11

12 **Example 5. Construction of the pDM16 C-terminal display**

13 **vector**

14

15 In order to demonstrate the display of peptides fused

16 to the C-terminus of a DBD on a PDCP a suitable vector,

17 pDM16, was created.

18

19 In pDM16 the pelB leader DNA sequence is fused directly

20 to the [oestrogen] <u>estrogen</u> receptor DBD sequence

21 removing the multiple cloning sites and the Gly$_4$Ser

22 linker DNA sequence found in pDM12 and pDM14, which are

23 appended to the C-terminal end of the DBD sequence

24 upstream of the HRE DNA sequence.

25

26 To create this vector two separate PCR reactions were

27 carried out on a Techne Progene thermal cycler for 30

28 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1

29 minute. Reaction products were electrophoresed on an

30 agarose gel, excised and products purified from the gel

31 using a Mermaid or Geneclean II kit, respectively,

1   according to the manufacturers instructions (Bio101, La

2   Jolla, California, U.S.A.).

3

4   In the first, the 5'-untranslated region and pelB

5   leader DNA sequence was amplified from 100ng of pDM12

6   vector DNA using 50pmol of each of the oligonucleotides

7   pelBFOR (SEQ ID No 33) (5'-CCTTGGCAGATTCCATCT

8   CGGCCATTGCCGGC-3') and M13REV (SEQ ID NO 27) (see

9   above) in a 100µl reaction containing 0.1mM dNTPs, 2.5

10 units Taqplus DNA polymerase, and 1x High Salt PCR

11 reaction buffer (20mM Tris-HCl pH 9.2, 60mM KCl, 2mM

12 $MgCl_2$) (Stratagene Ltd, Cambridge, U.K.).

13

14 In the second, the 3'-end of the pelB leader sequence

15 and the [oestrogen] estrogen receptor DBD was amplified

16 from 100ng of pDM12 vector DNA using 50pmol of each of

17 the oligonucleotides pelBBAK (SEQ ID No 34) (5'-CCGGCAA

18 TGGCCGAGATGGAATCTGCCAAGG-3') and pDM16FOR (SEQ ID No

19 35) (5'-TTTTGTCGACTCAATCAGTTATGCGGCCGCCAGCTGCAGG

20 AGGGCCGGCTGGGCCGACCCTCCTCCCCCAGACCCCACTTCACCCC-3') in a

21 100µl reaction containing 0.1mM dNTPs, 2.5 units

22 Taqplus DNA polymerase, and 1x High Salt PCR reaction

23 buffer (Stratagene Ltd, Cambridge, U.K.). Following gel

24 purification both products were mixed together and a

25 final round of PCR amplification carried out to link

26 the two products together as described above, in a

27 100µl reaction containing 0.1mM dNTPs, 2.5 units Taq

28 DNA polymerase, and 1x PCR reaction buffer (10mM Tris-

29 HCl pH 9.0, 5mM KCl, 0.01% Triton X®-100, 1.5mM $MgCl_2$)

30 (Promega Ltd, Southampton, U.K.).

31

1   The resulting DNA fragment, was HindIII-SalI

2   restriction enzyme digested and cloned into HindIII-

3   SalI digested pDM14 vector DNA as described in Example

4   1 to create pDM16. The sequence of pDM16 between the

5   HindIII and EcoRI restriction enzyme sites was checked

6   by DNA sequencing. The final vector sequence between

7   these two sites is shown in Figure 5 (see SEQ ID Nos 13

8   and 14).

9

10 **Example 6. Display of the C-terminal fragment of human**

11 **N-cadherin on the surface of a PDCP**

12

13 cDNA libraries of peptides can be constructed by many

14 methods known to those skilled in the art. One commonly

15 used method for constructing a peptide library uses

16 oligo dT primed cDNA, prepared from polyA+ mRNA. In

17 this method the first-strand synthesis is carried out

18 using an oligonucleotide which anneals to the 3'-end

19 polyA tail of the mRNA composed of T_n (where n is

20 normally between 10 and 20 bases) and a restriction

21 enzyme site such as NotI to facilitate cloning of cDNA.

22 The cDNA cloned by this method is normally composed of

23 the polyA tail, the 3'- end untranslated region and the

24 C-terminal coding region of the protein. As an example

25 of the C-terminal display of peptides on a PDCP, a

26 human cDNA isolated from a library constructed by the

27 above method was chosen.

28

29 The protein N-cadherin is a cell surface molecule

30 involved in cell-cell adhesion. The C-terminal

31 cytoplasmic domain of the human protein (Genbank

1 database accession number: M34064) is recognised by a

2 commercially available monoclonal antibody which was

3 raised against the C-terminal 23 amino acids of chicken

4 N-cadherin (SIGMA catalogue number: C-1821). The 1.4kb

5 human cDNA fragment encoding the C-terminal 99 amino

6 acids, 3'- untranslated region and polyA tail (NotI

7 site present at the 3'-end of the polyA tail) was

8 amplified from approximately 20ng pDM7-NCAD#C with

9 25pmol of each oligonucleotide M13FOR (SEQ ID No 26)

10 and CDNPCRBAK1 (SEQ ID No 29) (see above) in a 50µl

11 reaction containing 0.1mM dNTPs, 2.5 units Taqplus DNA

12 polymerase, and 1x High Salt PCR reaction buffer (20mM

13 Tris-HCl pH 9.2, 60mM KCl, 2mM MgCl$_2$) (Stratagene Ltd,

14 Cambridge, U.K.) on a Techne Progene thermal cycler for

15 30 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1

16 minute. Following gel purification and digestion with

17 SfiI and NotI restiction enzymes, the PCR product was

18 cloned into pDM16 using an analogous protocol as

19 described in Example 1.

20

21 Clones containing inserts were identified by ELISA of

22 96 individual PDCP cultures prepared as described in

23 Example 3. A Dynatech Immulon 4 ELISA plate was coated

24 with 1:250 diluted anti-pan cadherin monoclonal

25 antibody in 100µl /well PBS overnight at 4°C. The plate

26 was washed 3x200µl/well PBS and blocked for 1 hour at

27 37°C with 200µl/well 2% Marvel non-fat milk powder/PBS

28 and then washed 2x200µl/well PBS. 50µl PDCP culture

29 supernatant was added to each well containing 50µl/well

30 4% Marvel/PBS, and allowed to bind for 1 hour at

31 ambient temperature. The plate was washed three times

1 with 200µl/well PBS/0.1% Tween 20, then three times

2 with 200µl/well PBS. Bound PDCPs were detected with

3 100µl/well, 1:5000 diluted anti-M13-HRP conjugate

4 (Pharmacia) in 2% Marvel/PBS for 1 hour at ambient

5 temperature and the plate washed six times as above.

6 The plate was developed for 15 minutes at ambient

7 temperature with 100µl/well freshly prepared TMB

8 (3,3',5,5'-Tetramethylbenzidine) substrate buffer

9 (0.005% H_2O_2, 0.1mg/ml TMB in 24mM citric acid/52mM

10 sodium phosphate buffer pH 5.2). The reaction was

11 stopped with 100µl/well 12.5% H_2SO_4 and read at 450nm.

12 The nucleotide sequence of an ELISA positive clone

13 insert and DBD junction was checked by DNA sequencing

14 using oligonucleotides M13FOR (SEQ ID No 26) (see

15 Example 1) and ORSEQBAK (SEQ ID No 36) (5'-

16 TGTTGAAACACAAGCGCCAG-3').

17

18 A fifty-fold concentrated stock of C-terminal N-

19 cadherin PDCP particles was prepared by growing the un-

20 infected TG1 clone in 1ml 2xTY culture broth

21 supplemented with 1% glucose and 100µg/ml ampicillin

22 for five hours at 37°C, shaking at 200rpm and infecting

23 with 10^8 kanamycin resistance units (kru) M13K07 helper

24 phage at 37°C for 30 minutes without shaking, then for

25 30 minutes with shaking at 200rpm. Infected bacteria

26 were transferred to 20ml 2xTY broth supplemented with

27 25µg/ml kanamycin, 100µg/ml ampicillin, and 20µM IPTG,

28 then incubated overnight at 30°C, shaking at 200rpm.

29 Bacteria were pelleted at 4000rpm for 20 minutes in

30 50ml Falcon tubes, and 4ml 2.5M NaCl/20% PEG 6000 was

1   added to 20ml of PDCP supernatant, mixed vigorously and

2   incubated on ice for 1 hour to precipitate particles.

3

4   The particles were pelleted at 11000rpm for 30 minutes

5   in 50ml Oakridge tubes at 4°C in a Sorvall RC5B

6   centrifuge, then resuspended in PBS buffer after

7   removing all traces of PEG/NaCl with a pipette, then

8   bacterial debris removed by a 5 minute 13500rpm spin in

9   a microcentrifuge. The supernatant was filtered through

10 a 0.45µm polysulfone syringe filter. The concentrated

11 stock was two-fold serially diluted and used in ELISA

12 against plates coated with anti-pan-cadherin antibody

13 as described above (see Figure 6).

14

15 This example demonstrates the principle of C-terminal

16 display using PDCPs, that C-terminal DBD-peptide fusion

17 PDCPs can be made which can be detected in ELISA, and

18 the possibility that oligo dT primed cDNA libraries may

19 be displayed using this method.

20

21 **Example 7. Display of *in vivo* biotinylated C-terminal**

22 **domain of human propionyl CoA carboxylase on the**

23 **surface of a PDCP**

24

25 Example 6 shows that the C-terminal domain of human N-

26 cadherin can be expressed on the surface of a PDCP as a

27 C-terminal fusion with the DBD. Here it is shown that

28 the C-terminal domain of another human protein

29 propionyl CoA carboxylase alpha chain (Genbank

30 accession number: X14608) can similarly be displayed,

31 suggesting that this methodology may be general.

1

2 The alpha sub-unit of propionyl CoA carboxylase alpha

3 chain (PCC) contains 703 amino acids and is normally

4 biotinylated at position 669. It is demonstrated that

5 the PCC peptide displayed on the PDCP is biotinylated,

6 as has been shown to occur when the protein is

7 expressed in bacterial cells (Leon-Del-Rio & Gravel;

8 1994, J. Biol. Chem. 37, 22964-22968).

9

10 The 0.8kb human cDNA fragment of PCC alpha encoding the

11 C-terminal 95 amino acids, 3'- untranslated region and

12 polyA tail (NotI site present at the 3'-end of the

13 polyA tail) was amplified and cloned into pDM16 from

14 approximately 20ng pDM7-PCC#C with 25pmol of each

15 oligonucleotide M13FOR (SEQ ID No 26) and CDNPCRBAK1

16 (SEQ ID No 29) as described in Example 6.

17

18 Clones containing inserts were identified by ELISA as

19 described in Example 6, except that streptavidin was

20 coated onto the ELISA plate at 250ng/well, in place of

21 the anti-cadherin antibody. The nucleotide sequence of

22 an ELISA positive clone insert and DBD junction was

23 checked by DNA sequencing using oligonucleotides M13FOR

24 (SEQ ID No 26) and ORSEQBAK (SEQ ID No 36) (see above).

25 A fifty-fold concentrated stock of C-terminal PCC PDCP

26 particles was prepared and tested in ELISA against

27 streptavidin as described in Example 6 (see Figure 7).

28

29 This example shows not only that the peptide can be

30 displayed as a C-terminal fusion on a PDCP, but also

31 that *in vivo* modified peptides can be displayed.

1

2   **Example 8. Construction of a human scFv PDCP display**

3   **library**

4

5   This example describes the generation of a human

6   antibody library of scFvs made from an un-immunised

7   human. The overall strategy for the PCR assembly of

8   scFv fragments is similar to that employed by Marks, J.

9   D. et al. 1991, J. Mol. Biol. 222: 581-597. The

10   antibody gene oligonucleotides used to construct the

11   library are derived from the Marke et al., paper and

12   from sequence data extracted from the Kabat database

13   (Kabat, E. A. et al., Sequences of Proteins of

14   Immunological Interest. 4th edition. U.S. Department of

15   Health and Human Services. 1987). The three linker

16   oligonucleotides are described by Zhou et al. (1994,

17   Nucleic Acids Res., 22: 888-889), all oligonucleotides

18   used are detailed in Table 1.

19

20   First, mRNA was isolated from peripheral blood

21   lymphocytes and cDNA prepared for four repertoires of

22   antibody genes IgD, IgM, Igκ and Igλ, using four

23   separate cDNA synthesis primers. VH genes were

24   amplified from IgD and IgM primed cDNA, and VL genes

25   were amplified from Igκ and Igλ primed cDNA. A portion

26   of each set of amplified heavy chain or light chain DNA

27   was then spliced with a separate piece of linker DNA

28   encoding the 15 amino acids $(Gly_4 Ser)_3$ (Huston, J. S.

29   et al. 1989, Gene, 77: 61). The 3'-end of the VH PCR

30   products and the 5'-end of the VL PCR products overlap

31   the linker sequence as a result of incorporating linker

1 sequence in the JH, Vκ and Vλ family primer sets (Table

2 1). Each VH-linker or linker-VL DNA product was then

3 spliced with either VH or VL DNA to produce the primary

4 scFv product in a VH-linker-VL configuration. This scFv

5 product was then amplified and cloned into pDM12 as a

6 SfiI-NotI fragment, electroporated into TG1 and a

7 concentrated PDCP stock prepared.

8

9 **mRNA isolation and cDNA synthesis.**

10 Human lymphocyte mRNA was purified as described in

11 Example 2. Separate cDNA reactions were performed with

12 IGDCDNAFOR (SEQ ID No 37), IGMCDNAFOR (SEQ ID No 38),

13 IGKCDNAFOR (SEQ ID No 39) and IGλCDNAFOR (SEQ ID No 40)

14 oligonucleotides. 50pmol of each primer was added to

15 approximately 5μg of mRNA in 20μl of nuclease free

16 water and heated to 70°C for 5 minutes and cooled

17 rapidly on ice, then made up to a final reaction volume

18 of 100μl containing 50mM Tris pH 8.3, 75mM KCl, 3mM

19 $MgCl_2$, 10mM DTT, 0.5mM dNTPs, and 2000 units of

20 Superscript II reverse transcriptase (Life

21 Technologies, Paisley, Scotland, U.K.). The reactions

22 were incubated at 37°C for two hours, then heated to

23 95°C for 5 minutes.

24

25 **Primary PCRs.**

26 For the primary PCR amplifications separate

27 amplifications were set up for each family specific

28 primer with either an equimolar mixture of the JHFOR

29 primer set (SEQ ID Nos 41 to 44) for IgM and IgD cDNA,

30 or with SCFVκFOR (SEQ ID No 51) or SCFVλFOR (SEQ ID No

1   52) for IgK or Igλ cDNA respectively e.g. VH1BAK and

2   JHFOR set; Vκ2BAK (SEQ ID No 54) and SCFVκFOR (SEQ ID

3   No 51); Vλ3aBAK (SEQ ID No 66) and SCFVλFOR (SEQ ID No

4   52) etc. Thus, for IgM, IgD and Igκ cDNA six separate

5   reactions were set up, and seven for Igλ cDNA. A 50μl

6   reaction mixture was prepared containing 2μl cDNA,

7   25pmol of the appropriate FOR and BAK primers, 0.1mM

8   dNTPs, 2.5 units Taqplus DNA polymerase, and 1x High

9   Salt PCR reaction buffer (20mM Tris-HCl pH 9.2, 60mM

10   KCl, 2mM $MgCl_2$) (Stratagene Ltd, Cambridge, U.K.).

11   Reactions were amplified on a Techne Progene thermal

12   cycler for 30 cycles of 94°C, 1 minute; 60°C, 1 minute;

13   72°C, 2 minutes, followed by 10 minutes at 72°C. Fifty

14   microlitres of all 25 reaction products were

15   electrophoresed on an agarose gel, excised and products

16   purified from the gel using a Geneclean II kit

17   according to the manufacturers instructions (Bio101, La

18   Jolla, California, U.S.A.). All sets of IgD, IgM, IgK

19   or Igλ reaction products were pooled to produce VH or

20   VL DNA sets for each of the four repertoires. These

21   were then adjusted to approximately 20ng/μl.

22

23   **Preparation of linker.**

24   Linker product was prepared from eight 100μl reactions

25   containing 5ng LINKAMP3T (SEQ ID No 76) template

26   oligonucleotide, 50pmol of LINKAMP3 (SEQ ID No 74) and

27   LINKAMP5 (SEQ ID No 75) primers, 0.1mM dNTPs, 2.5 units

28   Taqplus DNA polymerase, and 1x High Salt PCR reaction

29   buffer (20mM Tris-HCl pH 9.2, 60mM KCl, 2mM $MgCl_2$)

30   (Stratagene Ltd, Cambridge, U.K.). Reactions were

1 amplified on a Techne Progene thermal cycler for 30

2 cycles of 94°C, 1 minute; 60°C, 1 minute; 72°C, 1

3 minute, followed by 10 minutes at 72°C. All reaction

4 product was electrophoresed on a 2% low melting point

5 agarose gel, excised and products purified from the gel

6 using a Mermaid kit according to the manufacturers

7 instructions (Bio101, La Jolla, California, U.S.A.) and

8 adjusted to 5ng/µl.

9

10 **First stage linking.**

11 Four linking reactions were prepared for each

12 repertoire using 20ng of VH or VL DNA with 5ng of

13 Linker DNA in 100µl reactions containing (for IgM or

14 IgD VH) 50pmol of LINKAMPFOR and VH1-6BAK set, or,

15 50pmol LINKAMPBAK and either SCFVκFOR (Igκ) or SCFVλFOR

16 (Igλ), 0.1mM dNTPs, 2.5 units Taq DNA polymerase, and

17 1x PCR reaction buffer (10mM Tris-HCl pH 9.0, 5mM KCl,

18 0.01% Triton X®-100, 1.5mM MgCl$_2$) (Promega Ltd,

19 Southampton, U.K.). Reactions were amplified on a

20 Techne Progene thermal cycler for 30 cycles of 94°C, 1

21 minute; 60°C, 1 minute; 72°C, 2 minutes, followed by 10

22 minutes at 72°C. Reaction products were electrophoresed

23 on an agarose gel, excised and products purified from

24 the gel using a Geneclean II kit according to the

25 manufacturers instructions (Bio101, La Jolla,

26 California, U.S.A.) and adjusted to 20ng/µl.

27

28 **Final linking and reamplification.**

29 To prepare the final scFv DNA products, five 100µl

30 reactions were performed for VH-LINKER plus VL DNA,

1 and, five 100μl reactions were performed for VH plus

2 LINKER-VL DNA for each of the four final repertoires

3 (IgM VH-VK, VH-Vλ; IgD VH-VK, VH-Vλ) as described in

4 step (d) above using 20ng of each component DNA as

5 template. Reaction products were electrophoresed on an

6 agarose gel, excised and products purified from the gel

7 using a Geneclean II kit according to the manufacturers

8 instructions (Bio101, La Jolla, California, U.S.A.) and

9 adjusted to 20ng/μl. Each of the four repertoires was

10 then re-amplified in a 100μl reaction volume containing

11 2ng of each linked product, with 50pmol VHBAK1-6 (SEQ

12 ID Nos 53 to 58) and either the JKFOR (SEQ ID Nos 66 to

13 70) or JλFOR (SEQ ID Nos 71 to 73) primer sets, in the

14 presence of 0.1mM dNTPs, 2.5 units Taq DNA polymerase,

15 and 1x PCR reaction buffer (10mM Tris-HCl pH 9.0, 5mM

16 KCl, 0.01% Triton X®-100, 1.5mM MgCl₂) (Promega Ltd,

17 Southampton, U.K.). Thirty reactions were performed per

18 repertoire to generate enough DNA for cloning.

19 Reactions were amplified on a Techne Progene thermal

20 cycler for 25 cycles of 94°C, 1 minute; 65°C, 1 minute;

21 72°C, 2 minutes, followed by 10 minutes at 72°C.

22 Reaction products were phenol-chloroform extracted,

23 ethanol precipitated, vacuum dried and re-suspended in

24 80μl nuclease free water.

25

26 **Cloning into pDM12.**

27 Each of the four repertoires was SfiI-NotI digested,

28 and electrophoresed on an agarose gel, excised and

29 products purified from the gel using a Geneclean II kit

30 according to the manufacturers instructions (Bio101, La

1 Jolla, California, U.S.A.). Each of the four

2 repertoires was ligated overnight at 16°C in 140µl with

3 10µg of SfiI-NotI cut pDM12 prepared as in Example 2,

4 and 12 units of T4 DNA ligase (Life Technologies,

5 Paisley, Scotland, U.K.). After incubation the

6 ligations were adjusted to 200µl with nuclease free

7 water, and DNA precipitated with 1µl 20mg/ml glycogen,

8 100µl 7.5M ammonium acetate and 900µl ice-cold (-20°C)

9 absolute ethanol, vortex mixed and spun at 13,000rpm

10 for 20 minutes in a microfuge to pellet DNA. The

11 pellets were washed with 500µl ice-cold 70% ethanol by

12 centrifugation at 13,000rpm for 2 minutes, then vacuum

13 dried and re-suspended in 10µl DEPC-treated water. 1µl

14 aliquots of each repertoire was electroporated into

15 80µl *E. coli* (TG1). Cells were grown in 1ml SOC medium

16 per cuvette used for 1 hour at 37°C, and plated onto

17 2xTY agar plates supplemented with 1% glucose and

18 100µg/ml ampicillin. 10^{-4}, 10^{-5} and 10^{-6} dilutions of the

19 electroporated bacteria were also plated to assess

20 library size. Colonies were allowed to grow overnight

21 at 30°C. Cloning into SfiI-NotI digested pDM12 yielded

22 an IgM-κ/λ repertoire of 1.16×10^9 clones, and an IgD-κ/λ

23 repertoire of 1.21×10^9 clones.

24

25 **Preparation of PDCP stock.**

26 Separate PDCP stocks were prepared for each repertoire

27 library. The bacteria were then scraped off the plates

28 into 30ml 2xTY broth supplemented with 20% glycerol, 1%

29 glucose and 100µg/ml ampicillin. 3ml was added to a

30 50ml 2xTY culture broth supplemented with 1% glucose

1 and 100µg/ml ampicillin and infected with 10^{11} kanamycin

2 resistance units (kru) M13K07 helper phage at 37°C for

3 30 minutes without shaking, then for 30 minutes with

4 shaking at 200rpm. Infected bacteria were transferred

5 to 500ml 2xTY broth supplemented with 25µg/ml

6 kanamycin, 100µg/ml ampicillin, and 20µM IPTG, then

7 incubated overnight at 30°C, shaking at 200rpm. Bacteria

8 were pelleted at 4000rpm for 20 minutes in 50ml Falcon

9 tubes, and 80ml 2.5M NaCl/20% PEG 6000 was added to

10 400ml of particle supernatant, mixed vigorously and

11 incubated on ice for 1 hour to precipitate PDCP

12 particles. Particles were pelleted at 11000rpm for 30

13 minutes in 250ml Oakridge tubes at 4°C in a Sorvall RC5B

14 centrifuge, then resuspended in 40ml water and 8ml 2.5M

15 NaCl/20% PEG 6000 added to reprecipitate particles,

16 then incubated on ice for 20 minutes. Particles were

17 again pelleted at 11000rpm for 30 minutes in 50ml

18 Oakridge tubes at 4°C in a Sorvall RC5B centrifuge, then

19 resuspended in 5ml PBS buffer, after removing all

20 traces of PEG/NaCl with a pipette. Bacterial debris was

21 removed by a 5 minute 13500rpm spin in a

22 microcentrifuge. The supernatant was filtered through a

23 0.45µm polysulfone syringe filter, adjusted to 20%

24 glycerol and stored at -70°C.

25

26 **Example 9. Isolation of binding activity from a N-**

27 **terminal display PDCP library of human scFvs**

28

29 The ability to select binding activities to a target of

30 interest from a human antibody library is important due

1 to the possibility of generating therapeutic human

2 antibodies. In addition, such libraries allow the

3 isolation of antibodies to targets which cannot be used

4 for traditional methods of antibody generation due to

5 toxicity, low immunogenicity or ethical considerations.

6 In this example we demonstrate the isolation of

7 specific binding activities against a peptide antigen

8 from a PDCP library of scFvs from an un-immunised

9 human.

10

11 The generation of the library, used for the isolation

12 of binding activities in this example, is described in

13 Example 8.

14

15 Substance P is an eleven amino acid neuropeptide

16 involved in inflammatory and pain responses *in vivo*. It

17 has also been implicated in a variety of disorders such

18 as psoriasis and asthma amongst others (Misery, L.

19 1997, Br. J. Dertmatol., 137: 843-850; Maggi, C. A.

20 1997, Regul. Pept. 70: 75-90; Choi, D. C. & Kwon, O.J.,

21 1998, Curr. Opin. Pulm. Med., 4: 16-24). Human

22 antibodies which neutralise this peptide may therefore

23 have some therapeutic potential. As this peptide is too

24 small to coat efficiently on a tube, as described in

25 Example 3, selection of binding activities was

26 performed in-solution, using N-terminal biotinylated

27 substance P and capturing bound PDCP particles on

28 streptavidin-coated magnetic beads.

29

30 **Enrichment for substance P binding PDCP particles.**

1   An aliquot of approximately 10^{13} a.r.u. IgM and IgD scFv

2   library stock was mixed with 1μg biotinylated substance

3   P in 800μl 4% BSA/0.1% Tween 20/PBS, and allowed to

4   bind for two hours at ambient temperature. Bound PDCPs

5   were then captured onto 1ml of BSA blocked streptavidin

6   coated magnetic beads for 10 minutes at ambient

7   temperature. The beads were captured to the side of the

8   tube with a magnet (Promega), and unbound material

9   discarded. The beads were washed eight times with 1ml

10   PBS/0.1% Tween 20/ 10μg/ml streptavidin, then two times

11   with 1ml of PBS by magnetic capture and removal of wash

12   buffer. After the final wash bound PDCPs were eluted

13   with 1ml of freshly prepared 0.1M triethylamine for 10

14   minutes, the beads were captured, and eluted particles

15   transferred to 0.5ml 1M Tris-HCl pH 7.4. Neutralised

16   particles were added to 10ml log phase TG1 *E. coli*

17   bacteria and incubated at 37°C without shaking for 30

18   minutes, then with shaking at 200rpm for 30 minutes.

19   10^{-3}, 10^{-4} & 10^{-5} dilutions of the infected culture were

20   prepared to estimate the number of particles recovered,

21   and the remainder was spun at 4000 rpm for 10 minutes,

22   and the pellet resuspended in 300μl 2xTY medium by

23   vortex mixing. Bacteria were plated onto 2xTY agar

24   plates supplemented with 1% glucose and 100μg/ml

25   ampicillin. Colonies were allowed to grow overnight at

26   30°C. A 100-fold concentrated PDCP stock was prepared

27   from a 200ml amplified culture of these bacteria as

28   described above, and 0.5ml used in as second round of

29   selection with 500ng biotinylated substance P. For this

1 round 100µg/ml streptavidin was included in the wash

2 buffer.

3

4 **ELISA identification of binding clones.**

5 Binding clones were identified by ELISA of 96

6 individual PDCP cultures prepared as described in

7 Example 3 from colonies recovered after the second

8 round of selection. A Dynatech Immulon 4 ELISA plate

9 was coated with 200ng/well streptavidin in 100µl /well

10 PBS for 1 hour at 37°C. The plate was washed

11 3x200µl/well PBS and incubated with 10ng/well

12 biotinylated substance P in 100µl /well PBS for 30

13 minutes at 37°C The plate was washed 3x200µl/well PBS

14 and blocked for 1 hour at 37°C with 200µl/well 2% Marvel

15 non-fat milk powder/PBS and then washed 2x200µl/well

16 PBS. 50µl PDCP culture supernatant was added to each

17 well containing 50µl/well 4% Marvel/PBS, and allowed to

18 bind for 1 hour at ambient temperature. The plate was

19 washed three times with 200µl/well PBS/0.1% Tween 20,

20 then three times with 200µl/well PBS. Bound PDCPs were

21 detected with 100µl/well, 1:5000 diluted anti-M13-HRP

22 conjugate (Pharmacia) in 2% Marvel/PBS for 1 hour at

23 ambient temperature and the plate washed six times as

24 above. The plate was developed for 10 minutes at

25 ambient temperature with 100µl/well freshly prepared

26 TMB (3,3',5,5'-Tetramethylbenzidine) substrate buffer

27 (0.005% H_2O_2, 0.1mg/ml TMB in 24mM citric acid/52mM

28 sodium phosphate buffer pH 5.2). The reaction was

29 stopped with 100µl/well 12.5% H_2SO_4 and read at 450nm.

30 Out of 96 clones tested, 10 gave signals greater than

31 twice background (background = 0.05).

1

2 **Characterization of a binding clone.**

3 A 50-fold concentrated PDCP stock was prepared from a

4 100ml amplified culture of a single ELISA positive

5 clone as described above. 10μl per well of this stock

6 was tested in ELISA as described above for binding to

7 streptavidin, streptavidin-biotinylated-substance P and

8 streptavidin-biotinylated-CGRP (N-terminal

9 biotinylated). Binding was only observed in

10 streptavidin-biotinylated-substance P coated wells

11 indicating that binding was specific. In addition,

12 binding to streptavidin-biotinylated substance P was

13 completely inhibited by incubating the PDCP with 1μg/ml

14 free substance P (see Figure 8). The scFv VH (SEQ ID

15 Nos 15 and 16) and VL (SEQ ID Nos 17 and 18) DNA and

16 amino acid sequence was determined by DNA sequencing

17 with oligonucleotides M13REV (SEQ ID No27) and ORSEQFOR

18 (SEQ ID No 36) and is shown in Figure 9.

19

20 The results indicate that target binding activities can

21 be isolated from PDCP display libraries of human scFv

22 fragments.

23

24 **Example 10**

25 In another example the invention provides methods for

26 screening a DNA library whose members require more than

27 one chain for activity, as required by, for example,

28 antibody Fab fragments for ligand binding. To increase

29 the affinity of an antibody of known heavy and light

30 chain sequence, libraries of unknown light chains

31 co-expressed with a known heavy chain are screened for

1 higher affinity antibodies. The known heavy chain

2 antibody DNA sequence is joined to a nucleotide

3 sequence encoding [a oestrogen] an estrogen receptor

4 DNA binding domain in a phage vector which does not

5 contain the [oestrogen] estrogen receptor HRE sequence.

6 The antibody DNA sequence for the known heavy chain (VH

7 and CH1) gene is inserted in the 5' region of the

8 [oestrogen] estrogen receptor DBD DNA, behind an

9 appropriate promoter and translation sequences and a

10 sequence encoding a signal peptide leader directing

11 transport of the downstream fusion protein to the

12 periplasmic space. The library of unknown light chains

13 (VL and CL) is expressed separately from a phagemid

14 expression vector which also contains the [oestrogen]

15 estrogen receptor HRE sequence. Thus when both heavy

16 and light chains are expressed in the same host cell,

17 following infection with the phage containing the heavy

18 chain-DBD fusion, the light chain phagemid vector is

19 preferentially packaged into mature phage particles as

20 single stranded DNA, which is bound by the heavy

21 chain-DBD fusion protein during the packaging process.

22 The light chain proteins are transported to the

23 periplasm where they assemble with the heavy chain that

24 is fused to the DBD protein as it exits the cell on the

25 PDCP. In this example the DBD fusion protein and the

26 HRE DNA sequences are not encoded on the same vector,

27 the unknown peptide sequences are present on the same

28 vector as the HRE sequence. Peptide display carrier

29 packages (PDCP) which encode the protein of interest

30 can then be selected by means of a ligand specific for

31 the antibody.

Table 1 (i) Oligonucleotide primers used for human scFv library construction

cDNA synthesis primers

IgMCDNAFOR	TGGAAGAGGCACGTTCTTTTCTTT
IgDCDNAFOR	CTCCTTCTTACTCTTGCTGGCGGT
IgκCDNAFOR	AGACTCTCCCCTGTTGAAGCTCTT
IgλCDNAFOR	TGAAGATTCTGTAGGGGCCACTGTCTT

JHFOR primers

JH1-2FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCAGGGTGCC
JH3FOR	TGAACCGCCTCCACCTGAAGAGACGGTGACCATTGTCCC
JH4-5FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCAGGGTTCC
JH6FOR	TGAACCGCCTCCACCTGAGGAGACGGTGACCGTGGTCCC

VH familyBAKprimers

VH1BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGGTGCAGTCTGG
VH2BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTCAACTTAAGGGAGTCTGG
VH3BAK	TTTTTGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGGTGGAGTCTGG
VH4BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTGCAGCTGCAGGAGTCGGG
VH5BAK	TTTTTGGCCCAGCCGGCCATGGCCGAGGTGCAGCTGTTGCAGTCTGC
VH6BAK	TTTTTGGCCCAGCCGGCCATGGCCCAGGTACAGCTGCAGCAGTCAGG

Light chain FOR primers

SCFVKFOR	TTATTCGCGGCCGCCTAAACAGAGGCAGTTCCAGATTTC
SCFVλFOR	GTCACTTGCGGCCGCCTACAGTGTGGCCTTGTTGGCTTG

VK family BAK primers

```
VK1BAK        TCTGGCGGTGGCGGATCGGACATCCAGATGACCCAGTCTCC
VK2BAK        TCTGGCGGTGGCGGATCGGATGTTGTGATGACTCAGTCTCC
VK3BAK        TCTGGCGGTGGCGGATCGGAAATTGTGTTGACGCAGTCTCC
VK4BAK        TCTGGCGGTGGCGGATCGGACATCGTGATGACCCAGTCTCC
VK5BAK        TCTGGCGGTGGCGGATCGGAAACGACACTCACGCAGTCTCC
VK6BAK        TCTGGCGGTGGCGGATCGGAAATTGTGCTGACTCAGTCTCC
```

JK FOR primers

```
JK1FOR        TTCTCGTGCGGCCGCCTAACGTTTGATTTCCACCTTGGTCCC
JK2FOR        TTCTCGTGCGGCCGCCTAACGTTTGATCTCCAGCTTGGTCCC
JK3FOR        TTCTCGTGCGGCCGCCTAACGTTTGATATCCACTTTGGTCCC
JK4FOR        TTCTCGTGCGGCCGCCTAACGTTTGATCTCCACCTTGGTCCC
JK5FOR        TTCTCGTGCGGCCGCCTAACGTTTAATCTCCAGTCGTGTCCC
```

Vλ family BAK primers

```
Vλ1BAK        TCTGGCGGTGGCGGATCGCAGTCTGTGTTGACGCAGCCGCC
Vλ2BAK        TCTGGCGGTGGCGGATCGCAGTCTGCCCTGACTCAGCCTGC
```

Table 1 (ii) Oligonucleotide primers used for human scFv library construction

```
Vλ3aBAK       TCTGGCGGTGGCGGATCGTCCTATGTGCTGACTCAGCCACC
      Vλ3bBAK TCTGGCGGTGGCGGATCGTCTTCTGAGCTGACTCAGGACCC
Vλ4BAK        TCTGGCGGTGGCGGATCGCACGTTATACTGACTCAACCGCC
Vλ5BAK        TCTGGCGGTGGCGGATCGCAGGCTGTGCTCACTCAGCCGTC
Vλ6BAK        TCTGGCGGTGGCGGATCGAATTTTATGCTGACTCAGCCCCA
```

Jλ primers

```
Jλ1FOR        TTCTCGTGCGGCCGCCTAACCTAGGACGGTGACCTTGGTCCC
Jλ2-3FOR      TTCTCGTGCGGCCGCCTAACCTAGGACGGTCAGCTTGGTCCC
```

Jλ4-5FOR   TTCTCGTGCGGCCGCCTAACCTAAAACGGTGAGCTGGGTCCC

Linker primers

LINKAMP3   CGATCCGCCACCGCCAGA

LINKAMP5   GTCTCCTCAGGTGGAGGC

LINKAMP3T   CGATCCGCCACCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCTGAGGAGAC